TAMPERE UNIVERSITY OF TECHNOLOGY

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AEROBIC BIOPROCESSES USING *ESCHERICHIA COLI*

Master of Science Thesis

Examiner: PhD Ville Santala
Examiner and topic approved in the
Faculty of Science and
Environmental Engineering Council
meeting on 9 November 2011

TIIVISTELMÄ

TAMPEREEN TEKNILLINEN YLIOPISTO
Biotekniikan koulutusohjelma
KANNISTO, MATTI: *Escherichia colia* käyttävät aerobiset bioprosessit
Diplomityö, 40 sivua
Helmikuu 2012
Pääaine: Ympäristöbiotekniikka (B)
Tarkastaja: FT Ville Santala
Avainsanat: *Escherichia coli*, fermentointi, bioprosessi, proteiinintuotto, korkea solutiheys, sokereidenkäyttö

Escherichia coli on eräs tutkituimmista mikro-organismeista ja sitä käytetään useissa bioteknologisissa sovellutuksissa. Näiden sovellutusten taloudellinen kannattavuus on pitkälti riippuvainen tuotettujen molekyylien saannoista. Koska *E. coli* on ollut suosittu kohde bakteriologisissa tutkimuksissa, on kertynyt paljon tietoa sen käyttöön perustuviin bioprosesseihin vaikuttavista tekijöistä. Tämän tiedon pohjalta on kehitetty malleja sen genetiikalle ja aineenvaihdunnalle, mikä on helpottanut bioprosessien saantoja lisäävien geenimuokkausten tekoa.

Tämä työ käsittelee tekijöitä, joilla on merkitystä *E. colin* käyttöön perustuvissa bioprosesseissa. Vaikka monet käsiteltävistä asioista ovat yleistettävissä useimpiin bioprosesseihin, proteiinintuottoon vaikuttavia tekijöitä käsitellään muita enemmän, sillä sen katsottiin olevan erityisen tärkeä osa bioprosessitekniikkaa. Työn alussa esitellään hyvin lyhyesti *E. colin* biologiaa. Sen jälkeen käsitellään bioprosesseihin vaikuttavat perusmuuttujat ja lopuksi selvitetään joitakin tuotteen muodostumiseen ja talteenottoon vaikuttavia tekijöitä. Kuten työstä käy ilmi, vaikka *E. coliin* pohjautuvia bioprosesseja on optimoitu merkittävästi, montaa niihin vaikuttavaa tekijää ei osata vielä optimoida riittävän hyvin. Täten on selvää, että tähän bakteeriin pohjautuvissa bioprosesseissa riittää vielä tutkittavaa pitkäksi aikaa.

ABSTRACT

TAMPERE UNIVERSITY OF TECHNOLOGY
Biotechnology study programme
KANNISTO, MATTI: Aerobic Bioprocesses Using *Escherichia coli*
Master of Science Thesis, 40 pages
February 2012
Major Subject: Environmental Bioengineering (B)
Examiner: PhD Ville Santala
Keywords: *Escherichia coli*, fermentation, bioprocess, protein production, high cell density, consumption of sugars

Escherichia coli is one of the most studied microorganisms and has found many uses in biotechnological applications. In order to make these applications economically feasible, it is necessary to get sufficiently high yields of the product molecules. Due to its popularity in bacteriological studies, a lot of knowledge about factors that are important to consider when optimizing *E. coli* bioprocesses has accumulated. This knowledge has been used to construct models about genetics and metabolism of this bacterium making genetic modification to improve bioprocess yields easier.

Factors important in bioprocesses using *E. coli* are discussed in this work. While most of the work is concentrated on basic properties common to most bioprocesses, special emphasis is put on bioprocesses related to protein production as it was considered to be one of the most important bioprocess application. First, a very brief introduction to biology of *E. coli* is given. Then the basic parameters' effects on bioprocesses are discussed, and finally some aspects of product formation and collection are discussed. It was shown in this work that, although a lot of progress has been made in optimization of bioprocesses where *E. coli* is used, in many aspects these processes still remain to be far from optimal. Therefore, it is clear that great amount of research on bioprocesses using this bacterium remains to be done.

PREFACE

This thesis was done as a part of Master of Science degree programme in biotechnology at Tampere University of Technology and is part of a project studying microbe-based industrial biotechnology. The laboratory work was carried out at the Environmental Engineering and Biotechnology laboratory at Tampere University of Technology. I would like to express my gratitude to professor Matti Karp, PhD Ville Santala and MsC Suvi Santala for supervising this work. Ville Santala and Suvi Santala designed most of the experiments and taught me the experimental protocols necessary for carrying them out. I am grateful to them for using so much of their time helping me as without their help I could not have completed the experimental work. I would also like to thank everyone working at the Environmental Engineering and Biotechnology laboratory for helping me with my experiments. MsC Nina Virolainen, who has taught me a lot about working in a laboratory, deserves special thanks. Finally, I want to thank my family for the support they have given me and my fellow students with whom I have had many stimulating talks about subjects related to this work.

Tampere, 23 February 2012

MATTI KANNISTO

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ABBREVIATIONS

ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid, cells' information storage molecule
GFP	Green fluorescent protein
HSL	N-3-oxohexanoyl-L-homoserine lactone
IPTG	Isopropyl β-D-1-thiogalactopyranoside
mRNA	Messenger RNA, an RNA used to transfer genetic information from DNA to the ribosomes
NADH	Nicotinamide adenine dinucleotide
RNA	Ribonucleic acid, a biomolecule involved in storage and expression of genetic information
rRNA	Ribosomal RNA, ribosomes' structural and functional part
tRNA	Transfer RNA, an RNA molecule used in protein synthesis

1. INTRODUCTION

The field of microbe-based biotechnology has become increasingly important for modern societies during last few decades (Demain 2000). Finding microorganisms suitable for biotechnological applications has been important for their successfulness. Many criteria must be met if an organism is to fulfill its role as a host organism in a bioprocess and, as all life forms have evolved to be as efficient as possible in passing on their own genetic material, they often are not optimal for these purposes. Thus cultivation conditions and often their genomes need to be modified. Many of the biotechnological applications are based on aerobic bioprocesses, in which most, if not all, chemical reactions are catalyzed by the host organism. One of the most popular microorganism in biotechnological applications is *Escherichia coli* which has been a workhorse in the study of bacterial biology and genetics (Madigan et al. 2009, pp. 279-281). The fact that lot is known about biology of *E. coli* and the ease of its cultivation and ability to grow on various hexose and pentose sugars have made it a bacterium of choice in many applications, for example, in production of valuable molecules from renewable resources (Aristidou & Penttilä 2000).

1.1. *Escherichia coli* Biology

Escherichia coli is a rod-shaped (Figure 1.1.1) (Goodell & Schwarz 1975), Gram-negative and facultatively anaerobic bacterial species which consists of numerous strains with diverse genomes containing lots of different genes (Lukjancenko et al. 2010). *E. coli* is an average-sized bacterium and is approximately 1 μm wide and 2 μm long (Madigan et al. 2009, p. 69). Its normal living environment is mammalian intestine where multiple strains of this bacterium can be found, each assumably evolved to thrive in its own ecological niche (Peekhaus & Conway 1998).

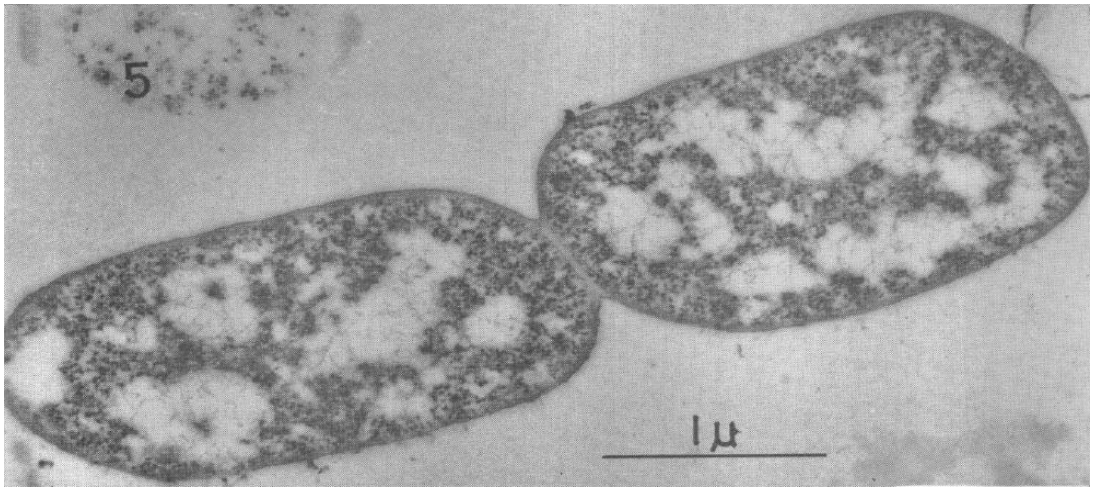

Figure 1.1.1. An electron micrograph of Escherichia coli at the late stage of cell division (Conti & Gettner 1962).

The genome of most studied *E. coli* strain, K-12, is about 4.6 Mb in length. 88 % of the genome code for open reading frames, 10 % for regulatory sequences, 1 % for tRNAs and rRNAs, and 0.5 % for repetitive sequences (Madigan et al. 2009, pp. 279-281). The genome contains genes on both of its strands although most highly expressed genes are oriented so that they are expressed in same direction as to which replication fork moves. Majority of the genome's genes are transcribed individually and thus are not part of any operon. It has been suggested, based on structure of its genome, that *E. coli* has acquired at least about one fifth of its genome from other species by lateral gene transfer, enabling it to live in environments otherwise unavailable (Lawrence & Ochman 1998). This seems also the most plausible explanation for the evolution of operons (Lawrence 1997) which play a crucial role in modern day bacteria. Thus it seems that history of *E. coli* has involved quite lot of 'genetic engineering' performed by blind forces of evolution, and should alleviate the fears experienced by many laymen about safety of man-made genetic alterations.

E. coli is a motile bacterium capable of moving in water with thin helical filaments (3.4 per cell) located in its periphery (Darnton et al. 2007). The movement of these filaments is mediated by motor proteins which can rotate the filaments in two directions. If the filaments rotate in counterclockwise direction (when viewed outside the cell), the cells are propelled forward, but if one of them rotates in clockwise direction, the cells tumble (Figure 1.1.2). The cell body always rotates in opposite direction of the filament bundle. This results in series of alternating onward movement and tumbling. The cell can direct its movement by regulating the length of onward movement and can thus move towards, or away from, increasing concentration of a molecule. The swimming speed of *E. coli* has been reported to be approximately 25 μm/s in a motility buffer (Darnton et al. 2007).

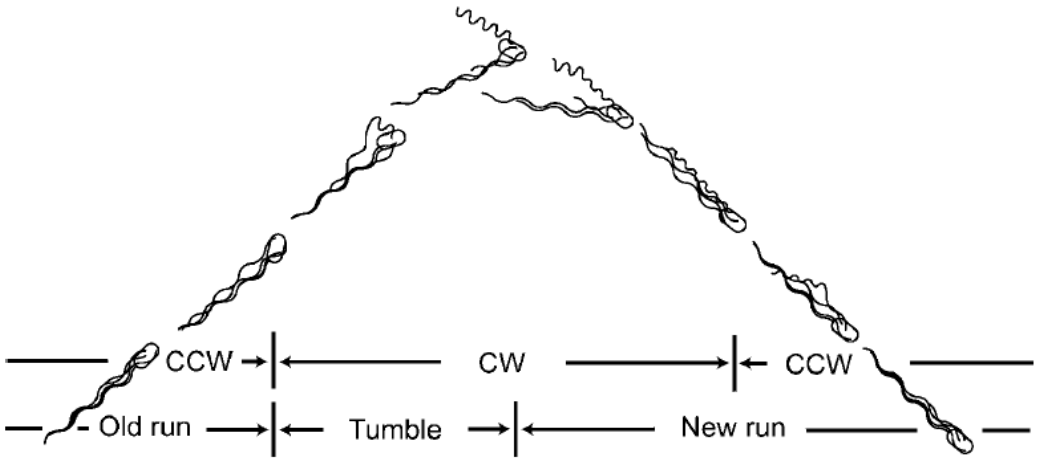

Figure 1.1.2. An idealized model for changing of taxis direction by an E. coli cell. The change in the direction is caused by a flagellum separated from the bundle. Abbreviations are: rotation of the flagellum to counterclockwise direction = CCW and to clockwise direction = CW (Adapted from Darnton et al. 2007).

Like all living entities, *E. coli* is a subject to evolutionary forces and its phenotype changes over generations to better cope with its living environment. In this process, mutations accumulate randomly in the genome of *E. coli* and cells with mutations allowing them to produce clones of themselves more efficiently than other cells increase their proportion in the population. Over long time scales the changes brought about by both random mutations and natural selection can lead to significant changes in metabolism, and thus in the biological fitness of *E. coli* cells. Such changes may include, for example, an ability to grow aerobically on citrate among other changes (Blount et al. 2008). The mutation elevated the cells fitness significantly, allowing them to grow to higher optical density in few generations (Figure 1.1.3). This ability to grow in citrate was brought about by mutations allowing uptake of citrate from the medium, a trait not present in the ancestral strain of *E. coli* used in the study. Unpredictable mutations are sometimes necessary to allow this kind of radical changes to happen because the trait is not easy to evolve. In any case, the example given above proves that, when using any microorganism in a bioprocess, evolution must be taken into account, especially if selective forces toward some phenotype are present in the bioprocess. Therefore, it is important to ensure that selective forces act not to cause the cells to evolve in a direction harmful to the bioprocess.

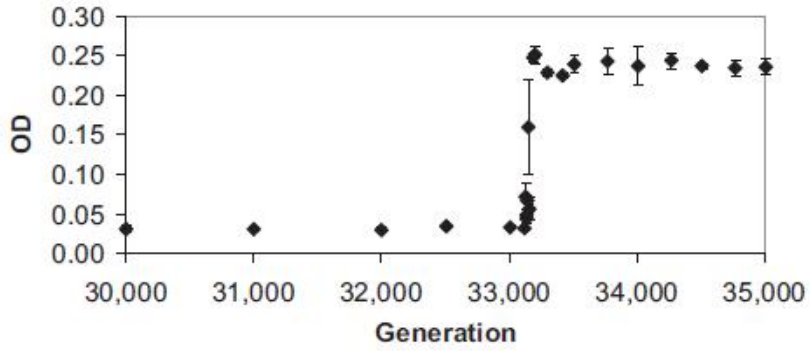

Figure 1.1.3. The increase in optical density of an Escherichia coli culture brought about by a mutation allowing the cells to grow on citrate anaerobically (Blount et al. 2008).

The vast amount of information on *E. coli* genetics available has favoured the selection of this microorganism as the host for many biotechnological applications. One of the most important applications using this bacterium as a host is production of recombinant proteins (Baneyx 1999). Because of its popularity in such applications during the early years of genetic engineering, lots of technological knowledge of its suitability in protein production and optimization of these processes has accumulated, further strenghtening its position as a number one protein production host choice. However, it is obvious that one organism can never be optimal for production of all proteins or overaccumulation of certain proteins. Because there has not been any reason for a bacterium like *E. coli* to evolve post-translational machinery capable of producing, for example, fully functional mammalian proteins, it may not be possible to easily engineer it to be ever able to produce such proteins. Also other differences between evolutionary distant organisms, such as different codon-usage between eukaryotes and prokaryotes, may make it difficult to achive efficient production of recombinant proteins in hosts such as *E. coli*. Nevertheless, many proteins can be produced in bacterial hosts and it is these proteins that should be possible to be produced in high enough amounts in *E. coli.* Also the fact that a lot is known about *E. coli* physiology, for example about protein folding and disulfide bond formation, makes it possible that many of the problems caused these physiological traits can be overcome (Baneyx & Mujacic 2004).

Also contributing to the attractiveness of *E. coli* as a host organism in fermentation technologies are different *in silico* models that have been constructed for it usually based on genetic or metabolic (Peekhaus & Conway 1998) information available. These include models as advanced as structural genomics (Matte et al. 2003) models. The structural genomics models apply the information gained from structural studies of *E. coli* proteome into the 'omics' analyses. These models contain information concerning structural properties of the enzymes of various metabolic routes, such as histidine biosynthesis and carbohydrate metabolism, thus complementing other systems biology models.

The cells can enter stationary phase if some of the essential nutrients are scarce (Peterson et al. 2005). Entry into stationary phase may occur in bioprocesses

intentionally or unintentinally. The limiting element may be carbon, nitrogen or phosphorus and the strategies for survival during the absence of the molecules containing these atoms vary with the limiting element. In the case of *E. coli*, the adaptation to the environment where an essential nutrient is limiting begins with a scavenging of this element. When the limiting nutrient is absent in the environment, *E. coli* cells adjust to these conditions by expressing the genes increasing their capability to withstand better these challenges. In addition, if carbon is limiting the cells express genes that enable them to use less abundant carbon sources more efficiently. The adjustment of *E. coli* to starvation conditions include changes in cell morphology. They become smaller and more spherical, increase of periplasmic osmoprotectant concentration and induce storage compound accumulation processes. Carbon and nitrogen starvation arrests the increase in cell size of *E. coli* but phosphorus starvation allows a slow increase in cell mass. Common to all these starvation conditions is that all starvation signals apparently affect the concentration or activity of the sigma factor RpoS, although in different ways (Figure 1.1.4). Phosphorus limitation increases the translation of its mRNA, carbon limitation decreases its protease-dependent degradation and nitrogen limitation apparently increases its activity posttranslationally.

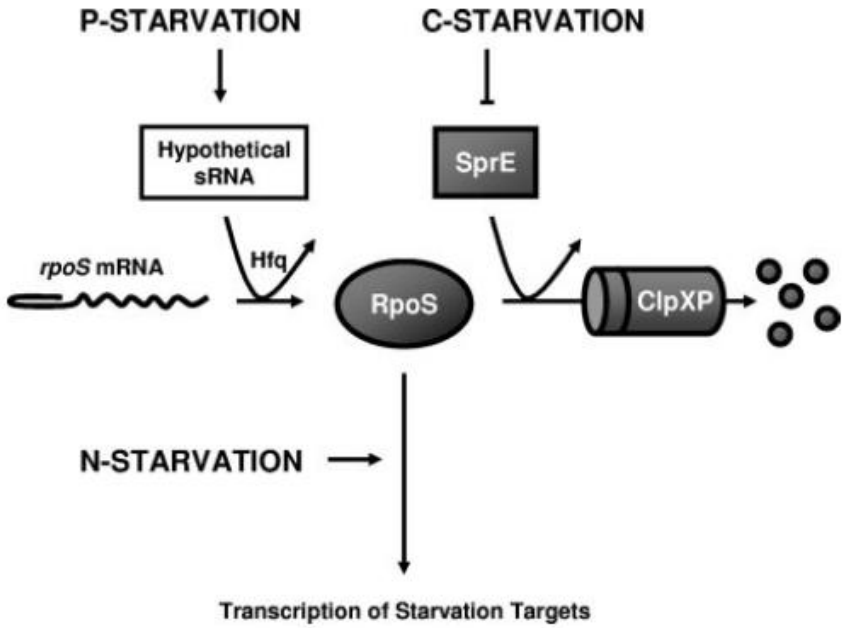

Figure 1.1.4. Different ways how RpoS activity is increased during phosphorus (P), carbon (C) or nitrogen (N) starvation (Adapted from Peterson et al. 2005).

This kind of nutrient-limited growth conditions should usually be avoided in fermentation of *E. coli*. However, it may prove valuable to understand how these conditions affect gene expression regulation as these changes could perhaps be exploited in metabolic engineering of *E. coli* to improve yields. Also, if storage compounds, such as glycogen or polyphosphate are to be produced, it may be

advantageous to induce starvation by limiting the supply of some essential nutrient to the cells.

1.2. Substrate Usage

In biotechnological applications where products are to be produced in great quantities, it is desirable that the organisms could grow on inexpensive substrates, especially if the end products are commodities. Such economically attractive materials include industrial wastes, waste waters and inedible parts of plants produced in agriculture and forest industry. The main part of plants unsuitable for other uses is lignocellulose, a structural material of plants, which consists of cellulose, hemicellulose and lignin (Kim et al. 2010). Of these polymers, cellulose consists of glucose monomers, while hemicellulose consists of a mixture of C5 (xylose, arabinose) and C6 (glucose, mannose, galactose) sugars depending on the plant species. Typically xylose, a C5 sugar, is dominant in the hemicellylose fraction. Lignin is a complex polymer of aromatic residues and is less attractive as carbon source in bioprocesses. Efficient bioprocesses require that, if there is a mixture of substrates in the growth medium, there should not be perturbations in the system caused when the organisms turn from metabolizing the most attractive substrate to less-attractive ones. Therefore, it would be beneficial if the microorganism used in such large-scale biotechnological applications could catabolize simultaneously all the sugars present in the medium. Most microorganisms however do preferentially catabolize glucose over other sugars. This trait has most likely evolved because glucose is the most abundant of sugars in most environments. Usually, if glucose is present, the transport of rarer sugars into the cytosol is hindered and the expression of genes needed for their catabolism is repressed. This phenomenon is called carbon catabolite repression and is mediated by cAMP level of the cells which is decreased by the presence of preferred substrates (Epstein et al. 1975). Catabolite repression is necessary to overcome if simultaneous consumption of various sugars in presence of glucose is to be achieved. Carbon catabolite repression inhibits uptake and catabolism of L-arabinose and D-xylose if D-glucose is present, but if L-arabinose and D-xylose are only sugars present, uptake and catabolism of D-xylose is repressed (Desai & Rao 2010). Desai & Rao (2010) showed that that the promoters used in expression of genes required for xylose and arabinose catabolism are repressed by glucose and arabinose or glucose, respectively (Figure 1.2.1). They also showed that, in the presence of arabinose, xylose consumption begins only after arabinose has been depleted in the medium (Figure 1.2.2). Thus, two layers of catabolite repression hinder efficient simultaneous utilization of D-glucose, L-arabinose and D-xylose.

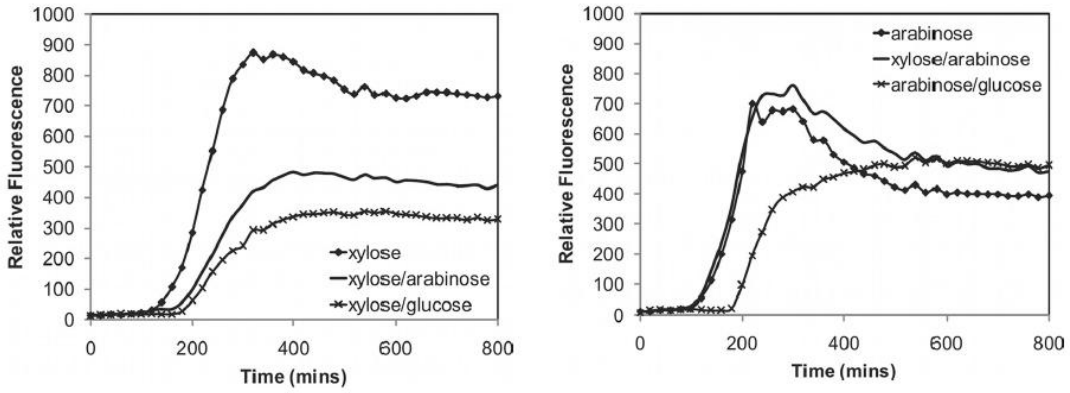

Figure 1.2.1. The effects of 2 mM concentrations of glucose, xylose and arabinose on activities of promoters P_{xylA} and P_{araB}, which are responsible for expression of genes required for xylose and arabinose catabolism, respectively, measured using a fluorescent protein as a reporter (Desai & Rao 2010).

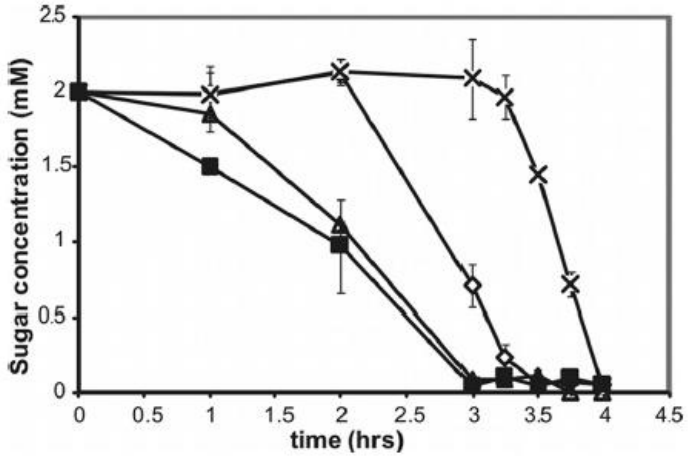

Figure 1.2.2. Concentration of arabinose when it was the only sugar used in the medium (solid triangles) or in the presence of 2 mM xylose (solid squares), and concentration of xylose used as the only sugar in the medium (rhombus) or in the presence of 2 mM arabinose (cross) (Desai & Rao 2010).

Microorganisms capable of consuming less attractive carbon sources such as arabinose and xylose have already been engineered. For example, an *E. coli* strain that can produce ethanol from glucose, arabinose and xylose with yields of around 90 % from the theoretical maximum has been constructed (Dien et al. 1998). The bacterium also produced similar ethanol yields with these three substrates alone or a mixture containing all of them. This kind of strains that can use these substrates are needed since many plant parts that cannot be used as food for human consumption are mainly composed of these sugars, and many organisms preferentially use glucose over arabinose and xylose or do not consume them at all which leads to dimished efficiency of the bioprocess. Because xylose is not as good carbon source as glucose, cells need to consume xylose in greater amounts than glucose to achieve same biomass, which may lead to higher conversion rate of xylose to fermentation product, although with lower growth rate of the bacteria (Kim et al. 2007).

Since one of the most important factors affecting the successfulness of a new technology is its economical feasibility, bioprocesses where valuable chemicals are produced are being optimized to be carried out using inexpensive substrates. This has been done with succinic acid producing *E. coli* (Agarwal et al. 2006). The bacterium was grown with cane molasses and corn steep liqour resulting in succinic acid production of 7.1 g/l in 36 hours of cultivation in 300 ml of medium. The process was further upscaled to 7.5 l medium where 17 g/l succinic acid production was achieved. The initial yield in unoptimized medium was 0.8 g/l in 60 hours of cultivation in 300 ml of medium. Thus a significant increase in yield was obtained by simply optimizing the medium used and bioprocess conditions. Replacing peptone with corn steep liqour increased succinate yield, most likely because the latter substrate contains richer variety of sugars and nitrogen-containing molecules. It is noteworthy that the above-mentioned study was carried out with wild type *E. coli* and the yield could be significantly increased by using *E. coli* strains genetically modified to produce succinic acid in greater quantities or microorganisms better suited for this application. By using such an organism, *Anaerobiospirillum succiniciproducens*, a yield of 24 g/l succinic acid has been obtained in a 32 hours of cultivation using wood hydrolysate as a carbon source and corn steep liqour as a nitrogen source. The wood hydrolysate contained approximately 20 g/l glucose and 7 g/l xylose. In the fermentation where the above-mentioned yield was obtained, the growth medium contained wood hydrolysate-based sugars equivalent of 27 g/l glucose and 10 g/l corn steep liqour. The use of wood hydrolysate gave similar succinate yields as had earlier been obtained with glucose as a carbon source. Also the replacement of yeast extract and polypeptone with corn steep liqour did not influence the results significantly. The results presented in the paper therefore indicate that, at least in this particular application, refined sugar and nitrogen sources can quite successfully be replaced with less expensive alternatives.

The examples presented above show that bioprocess optimization can be used to significantly increase the efficiency of bioprocesses where less expensive substrates are used, making them economically more feasible. Many of the attempts to use inexpensive substrates use wild type microorganisms and it is likely that the yields could be increased further if the organisms were subjected to metabolic engineering. The inexpensive substrates used in bioprocesses are often side products produced by other industries and are often of low value to the companies producing them. Their value is further lowered if they are classified as waste. If the producing capacity of these industries increases, the costs from processing these side products will probably increase too. Therefore, biotechnological applications using such substrates will become more attractive as they may be used to convert these materials into higher value chemicals. While many of the target molecules of fermentation technologies are currently produced by chemical means from oils, increasing oil prices will also contribute to this trend. However, a lot of work remains to be done in engineering the organisms being capable of using these substrates efficiently and optimizing the bioprocess conditions to allow more efficient product formation.

1.3. Cell Density

In most aerobic fermentation applications it is necessary to obtain high cell densities in order to achieve high product yields, especially if the product accumulates in cytosol. *E. coli* has been successfully grown to cell densities over 100 grams of dry weight per liter of medium (Lee 1996, Shiloach & Fass 2005). The maximum cell density that has been calculated to be possible to obtain with *E. coli* is about 200 grams of dry cell weigth per liter of growth medium. At cell densities higher than this, the growth medium would become highly viscous. Accumulation of acetate in high concentrations harms the fermentation process and makes it more difficult to reach high cell densities. Acetate forms in the medium when the specific growth rate of the cells is greater than the critical value (Eiteman & Altman 2006). When this occurs, the cells' oxygen consumption cannot keep up with subtrate usage and the substrate carbon flow is directed to acetate formation, which occurs in anaerobic conditions, regardless of the oxygen availability in the medium (Figure 1.3.1). This decreases the growth rate of the bacteria, the product yields as some of the substrate is used in this by-product formation, and accumulation of recombinant proteins.

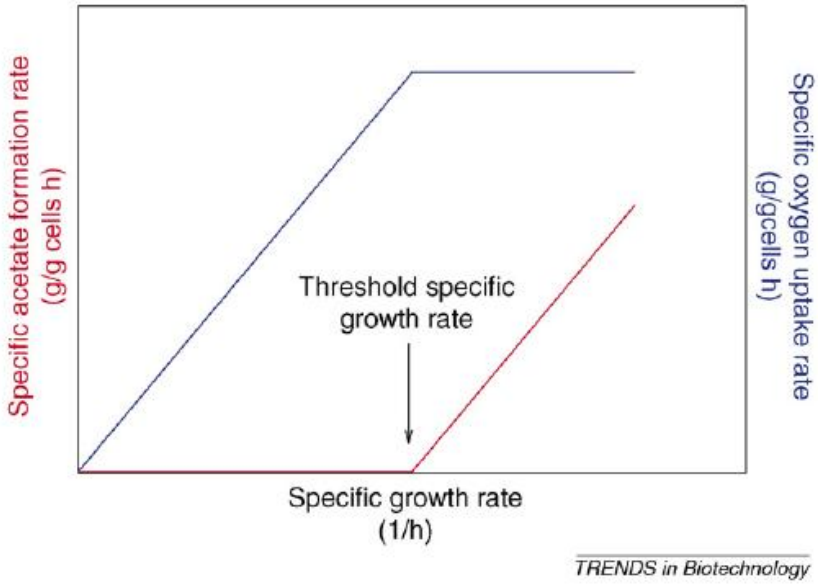

Figure 1.3.1. An idealized graph of specific acetate production and specific oxygen uptake rate as functions of specific growth rate (Eiteman & Altman 2006).

Acetate formation can be reduced or prevented, in addition to modifying the growth medium, by decreasing growth rate of the bacteria (Korz et al. 1995). In study by Korz et al. (1995), glucose and glycerol were used as substrates in a fed-batch fermentation to produce final dry cell weights of 128 and 148 g/l, respectively. The growth rates of the bacteria were kept at levels that do not induce acetic acid formation with predetermined substrate feeding rates without using expensive feedback systems. Thus, it was shown

that *E. coli* can be grown to high cell densities without excessive acetate production by simple and inexpensive means.

It has been shown that in *E. coli*, at least at high cell densities, the level to which acetate is produced is dependent on pH and glucose concentration of the growth medium. Acetate produced per dry cell weight decreased as the growth culture became denser, probably because acetate accumulated in the medium was consumed at higher rate. In the experiment, a sudden decrease in utilization of glucose and oxygen and production of CO_2 was observed at dry cell weight of approximately 30 g/l and acetate concentration of approximately 9.5 g/l when the pH of the growth medium was maintained at 7.0. At this point, the cells started to consume acetate from the medium, resulting in a increase in pH. When the acetate concentration of the culture had decreased to approximately half of the its highest value, glucose and oxygen consumption and CO_2 production rates resumed. When the fermentation was conducted at pH 6.5, the metabolic shift described above occurred approximately at values of 23 g/l and 6 g/l of dry cell weight and acetate concentration, respectively. Another shift occurred at this pH when the recovered culture had grown for a certain time. When the pH of the medium was further decreased to 6.0 the metabolic shift occurred at even lower dry cell weight and acetate levels. Furthermore, the cells did not recover from the change and succumbed after the shift. On the other hand, at pH 7.5 no such drastic change in metabolic state occurred, and acetate could accumulate at final concentration of 15 g/l (Kleman & Strohl 1994).

Alternatively the problem of acetate production can also be solved via metabolic engineering by removing either phosphotransacetylase or acetate kinase, the enzymes responsible for its production. Furthermore, it is possible to use another organism, which has been modified not to be able to use other substrates but acetate as a carbon source, to consume the acetate produced by the bacterium used in the fermentation. Furthermore, these high cell density cultivations have been subject to further improvement by, for example, development of strains used with the help of systems biology and development of better cultivation strategies (Choi et al. 2006).

Higher cell densities can also be obtained by enzymatically controlled release of the substrate (Krause et al. 2010). In study by Krause et al. (2010) *E. coli* could be grown to densities between 10 - 15 g/l and recombinant protein production could be significantly enhanced when compared to commonly used media. It should be noted, however, that the cells in the enzymatically controlled substrate release cultivation were grown for a longer time in order to achieve better recombinant protein yields and thus the cell densities obtained in the study are not directly comparable with each other.

High cell densities can be achieved also by simply optimizing growth conditions. For example, *Pseudomonas putida* has been grown to a density of 51 g/l in 40 hours of fed-batch cultivation by using optimized dissolved oxygen and ammonium ion concentrations and a limiting concentration of phosphorus (Diniz et al. 2004). *E. coli* has been grown to cell densities greater than 100 g/l by using medium optimized by computational means (Kishimoto & Suzuki 1995). In this approach, the medium was

modified by evaluating whether chemical components were present in the medium at sufficient concentrations and were added to it to more optimal concentrations. Amounts of different chemical elements in the cells were taken into account when optimizing the medium. Glucose concentration was also kept at low level to ensure that organic acids do not accumulate to the medium (Kishimoto & Suzuki 1995).

Certain bacteria can sense their own cell densities with quorum sensing systems (Bodman et al. 2008), a phenomenon that may be important to understand in optimizing high cell density cultivations of *E. coli*. A good example of such behavior is the induction of bioluminescence by a marine bacterium *Vibrio fischeri* (Figure 1.3.2). *V. fischeri* produces N-3-oxohexanoyl-L-homoserine lactone (HSL) which is excreted into the living environment of the bacterium. The concentration of this molecule increases as the cell density of the bacterium increases. When its concentration reaches a threshold value, HSL enters the cell and binds a protein repressing the expression of *luxCDABE* operon which is responsible for light production. Binding of HSL with this protein results in inhibition of expression of the gene encoding for it and simultaneously it increases expression of genes required for production of enzymes required in light production. Thus the light production is started only at high cell densities. However, unlike HSL, not all metabolites excreted to growth medium are quorum sensing signals and a caution must be taken when studying possible quorum sensing systems of bacteria (Winzer et al. 2002). There is evidence for cell density-based inhibition of growth in *E. coli* (Carbonell et al. 2002) which may be due to, in addition to quorum sensing signaling, direct cellular contact inhibition. The cells were shown to decrease their growth rates when the cell density was increased by removing supernatant after the culture was centrifuged. This should not alter concentrations of medium components or quorum sensing signaling molecules. Moreover, an increase in growth rates was observed when the cell density was decreased by diluting the medium, although the effect was less pronounced. Thus it may be possible to alter growth kinetics of *E. coli* by modifying the systems by which the cells sense their density without sensing excreted molecules, if such systems exist.

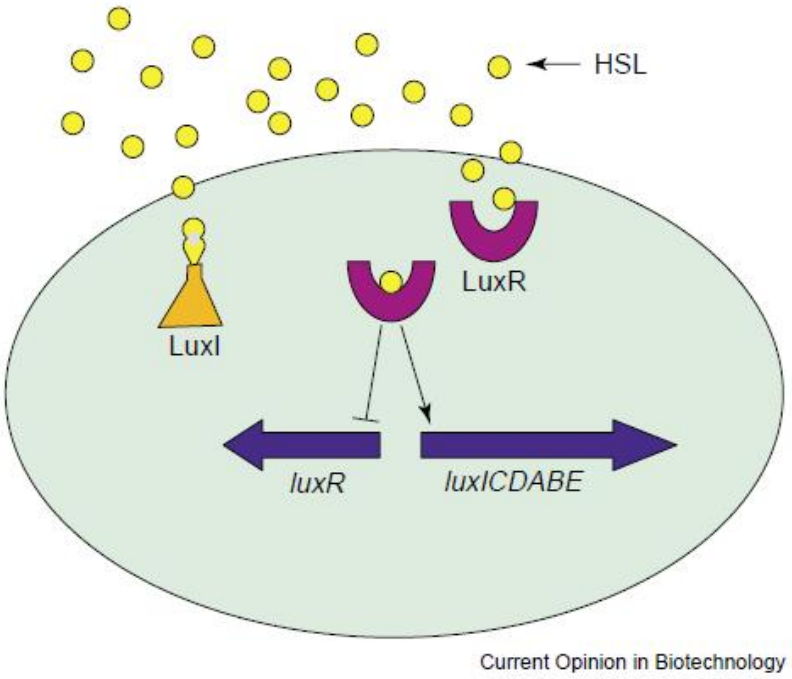

Figure 1.3.2. A schematic representation of quorum sensing system of Vibrio fischeri, a bioluminescent bacterium. HSL is N-3-oxohexanoyl-L-homoserine lactone, LuxI is an autoinducer synthase which produces HSL, LuxR is a transcription factor, luxR is a gene encoding for LuxR, and luxCDABE is an operon encoding for genes required in production of the light-producing enzyme, luciferase, and one of its substrate (March & Bentley 2004).

2. BIOPROCESSES

2.1. Bioprocess Monitoring

In bioprocesses many parameters such as temperature, cell density and pH have to be measured (Ritzka et al. 1997). The measurements should be easy to perform and should not disturb the cultivation process. Parameters such as temperature, pH and pO_2 can be measured *in situ* with sterilizable sensors while for measuring dry cell weight or concentrations of molecules in cell-free growth medium, samples have to be taken from the cultivation medium. Parameters that can be monitored online allow feedback control of the bioprocess and thus are important for its successfulness. Analysis of the data obtained from the measurements is also crucial for the control of the process. Several strategies with different levels of performance and computational difficulty exist (Figure 2.1.1). Proportional integral differential method simply adjust the parameter value to signal it obtains from bioreactor's sensors. It is a simple method and thus does not require much computational power but is very limited in controlling the bioprocess parameters. Fuzzy control methods allow more complex feed back loops to be used. It can be used to predict the behavior of the bioreactor with if-then rules. The most computationally demanding advanced control method can predict the behavior of the bioreactor even more precisely. It learns to recognize patterns in behavior of the bioprocess and is self-tuning.

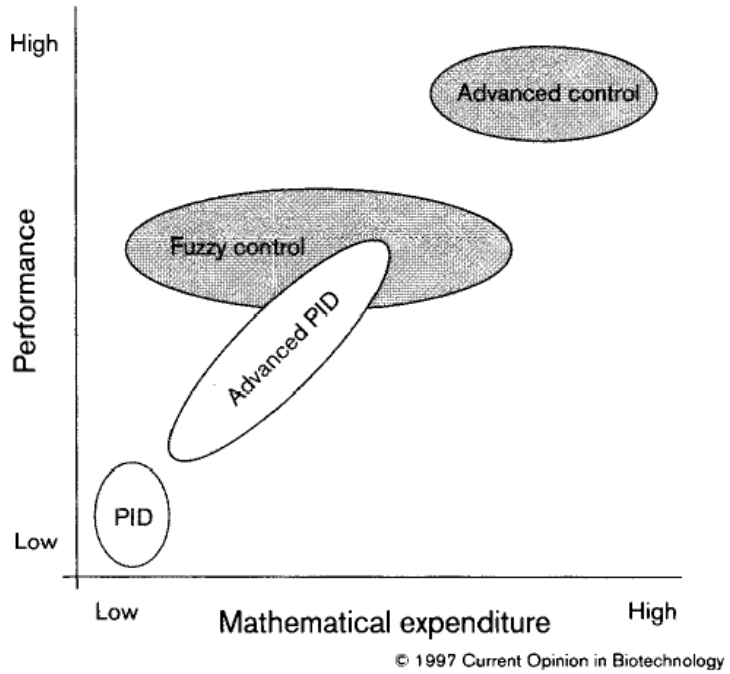

Figure 2.1.1. Comparison of performance and mathematical expenditure of different bioprocess parameter controlling strategies. PID stands for proportional integral differential (Ritzka et al. 1997).

Fluorescent compounds, which react with certain molecules in growth medium, and firefly luciferase (Inouye 2010), an enzyme with which ATP concentration can be correlated with light emission, can be used to gain information about bioprocesses growth medium (Ritzka et al. 1997). With firefly luciferase fused to glucose-sensing protein (Taneoka et al. 2009) it may also be possible to monitor glucose concentration in the bioprocess online. Bacterial *luxCDABE* operon and the gene for green fluorescent protein (GFP) (Tsien 1998) do not require addition of any substrate and can thus be easily used to monitor gene expression in the cultivated cells. This can be helpful in, for example, construction of genetic circuits possibly used in some microorganisms in bioprocesses. However, measurements taken from the biological systems always some degree of variance which may be at least partially caused by oscillatory processes in individual cells (Kruse & Jülicher 2005) that affect the cultivation as a whole. Measurements taken from the cultivations represent average behavior of the cells and there may be significant differences between individual cells. In one approach to optimize bioprocesses, cells' response to changed cultivation conditions, for example, temperature is measured from individual cells (Müller et al. 2000). With this approach, more accurate information can be obtained about things such as how temperature affects cell membrane fluidity or growth.

2.2. Aeration and Mixing

In aerobic bioprocesses, sufficient oxygen supply and equal distribution of nutrients are necessary if cells are to be grown to high densities which makes aeration and mixing

important parameters in bioprocesses. *E. coli* requires approximately 1 g of oxygen for increasing 1 g of cell biomass (Shiloach & Fass 2005). Supplying this amount of oxygen to the medium shouldn't be a problem in commonly used bioreactors, at least at laboratory scale, if growth rate of 0.2 h^{-1} and final biomass of 350 g/l are to be obtained. This goal could be achieved if the fermentor could supply pure oxygen at 1.5 M O_2 l/h at 25 ˚C which is usually possible with most fermentors. As 350 g/l is more than theoretical maximum cell density, it can be concluded that oxygen supply should not be the parameter restricting growth of *E. coli* cells in a bioreactor. However, dissolved oxygen content of the medium is not uniform in fermentations and some parts of the medium usually are not sufficiently aerated, even though the fermentor should theoretically be able to supply enough oxygen. If oxygen partial pressure decreases in the medium it can be restored by increasing mixing speed (Lee 1996). Increasing mixing speed can however be limited by efficiency of the fermentor, especially in large fermentors. *E. coli* starts to produce acetate and other organic acids if it lives in oxygen-limited medium (Enfors et al. 2001) so insufficiently aerated regions' formation in fermentor should be avoided. Sufficient mixing is important also to ensure that nutrients fed to the medium will be spread equally in the medium. Usually the cells that are close to nutrient feeding injector have access to much higher concentrations of nutrients than cells that are located farther from the injector. Pure oxygen is more expensive than air which is why it is not often used.Thus it would be beneficial if normal air could be used. This can be achieved by lowering medium's temperature (Lee 1996) which increases the amount of dissolved oxygen. It is also possible to increase the amount of oxygen dissolved in growth medium by increasing the pressure of the gas phase in the fermentor (Thiry & Cingolani 2002). Increasing pressure also increases solubility of other gases, such as CO_2, in the growth medium whose effects have to be taken into account when using this approach. Also aeration timing and rate have to be considered when optimizing a bioprocess (Lin et al. 2011).

The effects of other gases on the fermentation's success also have to be considered. For example, it has been shown that CO_2 concentration affects fermentative succinate production in metabolically engineered *E. coli* under anaerobic conditions (Lu et al. 2009). It was found out that, by increasing the concentration of CO_2 in the gas phase of the fermentor, a change from 1.9 to 225 mg/g h in specific succinate productivity occurred. Production of succinate requires carboxylation of phosphoenol pyruvate to oxaloacetate which is, like succinate, a citric acid cycle intermediate and can thus be used in succinate biosynthesis. This carboxylation reaction consumes CO_2 which explains the need for its increased concentration in the gas phase for improved succinate production, and, rather surprisingly, increased flux to pentose phosphate pathway which produces CO_2. The results of the study indicated that at low (< 3 %) CO_2 concentrations mass transfer to the cells limits the succinate production while this was due to rate of bicarbonate formation from CO_2 at intermediate (10 - 30 %) concentrations and due to enzymatic activity of phosphoenol pyruvate carboxylase at high (> 50 %) CO_2 concentrations (Lu et al. 2009). Thus, it is obvious that a profound understanding of *E.*

coli metabolism and optimization of fermentation conditions with regard to all parameters are necessary if the highest product yields are to be achieved.

Parameters such as gravity and shear stress can have unexpected effects on the behavior of the cells in the bioprocess. In an application where microcin B17, an antibacterial polypeptide, was produced in *E. coli*, growing the cells in simulated microgravity allowed the cells to grow to higher density while microcin B17 production decreased (Fang et al. 1997). The fermentor capable of producing simulated microgravity subjected the cells to much lower shear stress than common shake flasks and this lowered shear stress was found to change the microcin B17 from being accumulated in cytosol to be secreted into the medium. This example shows that shear stress experienced by the cells can have unexpected effects on the bioprocess. Also gravity, a parameter that is usually not considered in optimizing bioprocesses, can affect the yield and cell density obtained. While this parameter is certainly not very important to be considered in most cases as most fermentors cannot generate conditions for simulated microgravity, it may become more important in future if fermentors capable of such feat became more popular. It is also important to consider the cost of generating simulated microgravity or reducing shear stress. It may not be economically feasible to generate such conditions, even if they increased the yield, if they increased the cost of running the bioprocess significantly. This will probably be the case especially when large scale bioreactors are used. Nevertheless, in smaller bioreactors where high-value products are produced with more fragile cells, such as insect cells, these effects may become more important to consider.

2.3. Removal of the Inhibitors of Growth

For the removal of small molecular weight compounds from the medium, produced by the bacteria during the fermentation, a dialysis method has been developed. This fermentation method using recombinant strain of *E. coli* has been scaled up successfully with dialysis modules external to the fermentor (Figure 2.3.1) (Fuchs et al. 2002). In the manuscript by Funchs et al. also a dry cell density of 220 g/l, a value close to the theoretical maximum which is limited by viscosity of the culture, was described in a 2 l fed-batch fermentation with dialysis and oxygenation of the dialysate after less than 30 h of fermentation. In the same conditions without dialysis less than 50 g/l of dry cell weight was obtained. Thus, it is clear that the removal of growth-inhibiting compounds by dialysis or other method can be used effectively to achieve very high cell density in bioprocesses. The fact that this dialysis was performed also with 300 l fermentor indicates that it may be applicable at industrial scale. In the experiment, also recombinant protein production was studied with the fermentor system capable of producing abovementioned very high cell density. When the cells were induced to produce this protein they grew to a density of approximately 140 g/l after about 30 h of fermentation and gave almost fourfold concentration of the recombinant protein

compared to typical industrial yields at the time of publication. Similar values were obtained in technical-scale fermentor of 300 l (Fuchs et al. 2002).

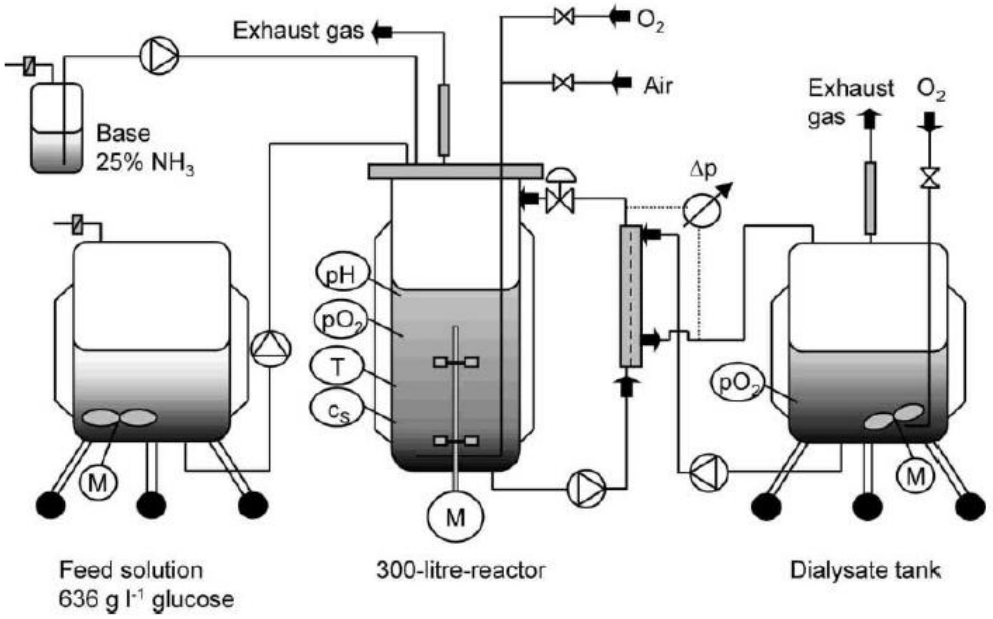

Figure 2.3.1. A schematic representation of the fermentor and its external modules used in the dialysis fermentation experiment carried out by Fuchs et al. (2002).

Dialysis of inhibitory molecules from the medium removes also nutrients (Eiteman & Altman 2006) which inevitably decreases the ratio of mass unit of product produced per mass unit of substrate added. Furthermore, dialysis approach does not decrease the rate of acetate produced and thus does not decrease the amount of substrate wasted in by-product formation. A more efficient approach in this aspect would be decreasing or eliminating the acetate formation in the first place.

2.4. Feeding Rate of the Substrate and Growth Rate

In order to minimize the accumulation of fermentation side products that may decrease the cell density obtained, it is necessary to keep the growth rate of the bacteria at low enough levels. This is normally achieved by adjusting the rate at which substrate is fed to the reactor. However, it is a prerequisite for successful adjustment of feeding rate that the actual growth rate of the bacteria can be measured.

The exothermic nature of catabolic reactions causes microorganisms used in fermentation to produce heat as their biomass increases. This can be exploited in controlling the growth rate of the bacteria by altering the feeding rate of substrate based on the information of their growth gathered from heat produced during the fermentation (Biener et al. 2010). In this manuscript, a calorimetric control system was described for maintaining a growth rate of the bacteria below the critical value at which growth-inhibiting side products, such as acetate, start to accumulate. With this system it was

shown that the growth rate could be measured from the heat production of the fermented bacteria without recombinant protein overexpression quite successfully and with GFP overexpression less accurately. While growth rate was set to 0.2 h^{-1} at fed phase of the fermentation, mean apparent growth rate of 0.17 ± 0.03 h^{-1} and mean real growth rate of 0.16 ± 0.03 h^{-1} were measured for fermentation without recombinant protein production. These values were 0.20 ± 0.04 h^{-1} and 0.24 ± 0.03 h^{-1} before the induction of GFP overexpression and 0.19 ± 0.05 h^{-1} and 0.10 ± 0.03 h^{-1} after its induction. The fact that growth rate of the bacteria could not be measured accurately when GFP was overexpressed was probably due to heat generated in recombinant protein production. Nevertheless, such a control system provides a relatively easy method for adjustment of growth rate with easy-to-perform measurements. The fact that increase in fermentor size increases the heat production by metabolism of the microorganisms per fermentor surface further makes this system more attractive for industrial-scale fermentors which usually are of large volume (Biener et al. 2010).

2.5. Fermentation Scale Up

In order to be economically feasible, bioprocesses often need to be scaled up so that higher amounts of the product can be obtained. Scaling up these processes make it more difficult to control certain parameters, such as temperature, amount of dissolved oxygen and pH of the medium, and these changes have to be taken into account when optimizing the scale-up. These challenges in scaling up a fermentation process arise mainly from the formation of zones with different conditions in large fermentors. These include areas of different substrate concentrations and oxygen concentration, which may contribute to mixed acid fermentation in the bacterial culture leading to growth inhibition (Enfors et al. 2001). This may be the reason for lowered yields in upscaled bioreactors and therefore should be avoided. These concentration differences can also cause stress and metabolic responses in the cells (Enfors et al. 2001). Therefore, scaling up may affect bioprocess in many ways at physiological level in addition to reducing biomass yields. The scale up process optimization thus requires cooperation of people working in various scientific and engineering fields as the things that have to be taken into account are numerous (Thiry & Cingolani 2002). Not all changes on bioprocess brought about by scaling up are harmful. Cultivating cells in large reactors can, for example, allow the cells to retain their capability to divide at growth rates close to 0.05 h^{-1} and remain healthy in longer cultivations, both of which do not occur at smaller reactors (Enfors et al. 2001).

When designing scaled-up bioprocesses, it is more important to consider the costs of controlling different variables of the fermentor than if the bioprocess was done at laboratory scale, where the processes do not have to be cost-effective. It is also important that the scaling up of the bioprocess does not impede the production of the desired product. The fermentative production of recombinant antigen K88 has been successfully scaled up from 5 l to 50 l reactor while maintaining similar growth rates

and protein yields as in the smaller scale bioreactor (Wong et al. 2003). Thus, the fermentor parameters could be optimized in small scale fermentations and these parameters gave good results in a larger scale fermentation, even though the larger fermentation was still much smaller than economically feasible large scale fermentations usually are.

A problem often encountered in large scale fermentations is contamination of the medium, which has been identified as one of the most common reasons for failure of the bioprocess. Contamination of fermentation medium can cause problems such as degradation of the product, production of unwanted end products from the substrate and change in fermentation conditions. The chance of contamination can be decreased by using temperatures lower than 20 ˚C or higher than 40 ˚C, pHs lower than 5 or higher than 8, very high or low concentrations of various medium components, such as sugars or trace metals, using antibiotics or using defined medium instead of rich one. Also the bioreactor design affects greatly the probability of contamination. The fermentation parameters, such as medium components, define what kind of contaminants the fermentation is prone to. The probability of contamination is lower for bioprocesses where one or more parameters do not allow growth of most contaminants but allow growth of the product-forming organism (Junker et al. 2006).

3. PRODUCT FORMATION AND COLLECTION

3.1. Protein Production

Recombinant protein production is one of the most economically and scientifically important applications where *E. coli* is used. A wide variety of proteins has been produced in this organism (Table 3.1.1), showing that it is a suitable host for production of proteins for various applications. Because of this, knowledge about *E. coli*'s protein production machinery is of utmost importance to both biotechnology industry and academic world. This knowledge is useful also in bioprocesses in which metabolically engineered *E. coli* is used to produce some fermentation product, as the foreign gene products must be expressed at suitable level and must not misfold. Usually it is necessary to produce the recombinant protein in very high proportions of dry cell weight of the host organism. This kind of protein accumulation is practically never advantageous to the host organism and often causes enough damage to the cells to harm the effectivity of the fermentation process. Therefore, it may be necessary to optimize the genetic elements used in the overexpression of the protein. This may include choosing between different inducers of the expression or different expression vectors, which affect the fitness of the host organism to different level. If the presence of the plasmid is of disadvantage to the organism, natural selection will favour the organisms lacking this genetic entity. This in turn leads to decrease in the proportion of the plasmid-hosting organisms in the population which affects adversely the target protein yield. The selection against plasmid hosting in the population can be fought by introducing antibiotic resistance-conferring genes to the plasmid and supplying the growth medium with appropriate antibiotics. As most antibiotics are quite expensive and are easily degradable in the usual growth conditions, they are not so attractive alternative for this counter-selection. Other strategies may employ for example genetic elements, such as genes for amino acid synthesis that are required for the survival of the organism, introduced in the overexpression construct or vector. This strategy has been employed in a *E. coli* strain unable to grow anaerobically by using a plasmid complementing the disrupted enzymatic activities which allows the bacterium to thrive under anaerobic conditions (Hespell et al. 1996). Thus, the cells do not get rid of the plasmid if they are grown anaerobically. However, if the cultivation is conducted under aerobic conditions, the plasmid is rapidly lost. This kind of complemention allows one to use foreign gene-bearing plasmids in fermentation applications without the need for expensive antibiotics.

Table 3.1.1. Proteins produced in various strains of E. coli in high cell density cultivations (adapted from Choi et al. 2006).

Products	Culture condition and carbon source	Productivity and characteristics
Insulin-like growth factor-2 (IGF-2)	pH-stat, R medium, glucose	9.69 g/L, inclusion body
Single-chain antibody variable fragment	Exponential feeding, defined medium, glucose	1.2 g/L, PelB signal sequence
Human interferon-γ (hIFN-gamma)	Exponential feeding, M9 modifed medium, glucose	2×10^7 U/mg protein, inclusion body
Human Interleukin-7	Large scale batch fermentation (1000L), semi-defined medium, glycerol	46% of total proteins, inclusion body
Phytase	DO-stat, glucose mineral salt medium	120 U/mL, *kil* gene coexpression
Carbamoylase	pH-stat, synthetic medium, glucose	14256 U/L, thermoreglulated T7 promoter
Human epidermal growth factor	Batch, semi-defined medium, glucose, lactose	242 mg/L
Human epidermal growth factor	pH-stat, MMBL medium, glucose	325 mg/L, OmpA signal sequence
Alkaline phosphatase	pH-stat, modified R medium, glucose, YE	5.2 g/L, Endoxylanase signal sequence.
Human granulocyte colony-stimulating factor (GCSF)	pH-stat, modified R medium, glucose	22% of total proteins, Endoxylanase signal sequence
Protective antigen protein	Batch, semi-defined medium, glycerol	125 mg/L, inclusion body, constitutive expression
Bone morphogenetic protein 2	Exponential feeding, defined medium, glucose	8.6 g/L, inclusion body.
Human mini-proinsulin	pH-stat, semi-defined medium, glucose	7 g/L, two stage cyclic fed-batch culture
Human interferon-α	Exponential feeding, defined medium, glucose	4 g/L, inclusion body
Animolevulinate synthease	Batch fermentation, LB medium, media optimized supplement	5.2 g/L
Annexin-V-hirudin chimeric protein	DO-stat, LB medium, glucose	10 mg/L/OD_{600} (after purification)
Human Tissue-type plasminogen activator	Exponential feeding, semi-defined medium, glucose, casein, YE	180 μg/L (after purification), StII signal sequence, DsbC coexpression
Human necrosis factor-related apoptosis-inducing ligand	Combined feeding using pH- and DO-stat, semi-defined medium, glucose	1.4 g/L soluble protein
Antifungal peptides	DO-stat, glycerol minimal salt medium	40% of total proteins, PelB signal sequence, not secreted, inclusion body
Human necrosis factor-α	Batch fermentation, semi-defined medium, glycerol	Constitutive production, P_{HCE} promoter
Bioadhesive protein	pH-stat, exponential, constant feeding, R medium, glucose	5.3 g/L
Human leptin	pH-stat, modified R medium, glucose, YE	41% of total proteins, endoxylanase signal sequence, DsbA coexpression
Pectate lyase	DO-stat, FB synthetic medium, glucose, casamino acid	2200 U/mL, PelB signal sequence
Human leptin	pH-stat, modified R medium, glucose, casamino acid	9.7 g/L, constitutive production, P_{HCE} promoter
Insulin-like growth factor-1 fusion protein	pH-stat, modified R medium, glycerol, YE	4.3 g/L, PrsA and GlpF coexpression

The presence of plasmids in the host cell, in addition to decreasing growth rates, final biomass obtained and glucose consumption and prolonging lag phase, changes its metabolism-related enzymes' expression levels (Ow et al. 2006). These changes include downregulation of biosynthetic and energy-production proteins' genes, such as glycolytic genes, and upregulation of heat shock protein-encoding genes. However, the differences in expression levels between plasmid-bearing cells and cells that bore no plasmid were less than two-fold. The presence of plasmid did not change expression of pentose phosphate pathway's genes which usually are expressed constitutively. High-copy-number plasmids usually produce more protein in the cells but this is not always the case (Makrides 1996). Furthermore, if the protein production is controlled by a strong promoter in such a plasmid, the cells' viability may be decreased. The promoter used should be strong but with a low level of basal expression level, meaning that expression of the protein should be minimal when not induced. This is especially important if the protein to be produced is toxic to the cells. The inducer used to start the protein production should be as inexpensive as possible. Thus, for example, the commonly used inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) is not considered to be a good inducer in large scale fermentations, and could be replaced by thermally activated *lac* repressor induction. It is also important that there are efficient transcription terminators downstream of the target gene to prevent expression of other genes of the plamid and upstream of the promoter used to minimize background expression of the target gene. The protein yield is also affected by translational effectiviness and stability of the produced mRNA.

It is also important for the successfulness of the protein production that the target proteins are folded correctly and that they do not form aggregates (Baneyx & Mujacic 2004). Translation rate and rate of protein folding have been recognized as major factors affecting aggregate formation in bacteria (Esposito & Chatterjee 2006). The solubility of the recombinant protein can be enhanced with molecular chaperones that promote correct folding of the protein produced, culturing the cells at lowered temperatures, selecting strains with reduced ability to aggregate the proteins, modification of the proteins, or modification of the growth medium (Makrides 1996). Solubility can also be enhanced by using fusion tags (Figure 3.1.1) which can also be used as affinity tags in purification of the protein (Esposito & Chatterjee 2006). The tags usually have to be removed, however, and their presence may affect the activity of the proteins. Sometimes it may be advantageous to let the overexpressed gene product to misfold and form inclusion bodies. For example, if the protein is toxic in its active form to the host organism or can be refolded easily, it may be easier to let these aggregates, which are relatively free from impurities such as other proteins, to form and refold them after purification. However, purification of proteins from aggregates is not without problems (Makrides 1996). The protein may not fold into its biologically active form and the total yield of the active protein may decrease. The protein yield is also affected by codon usage of the host organism. If the inserted DNA contains codons not favored by the host

organism the yield will be lowered. Thus, it may be necessary to modify the codons of the transgene to better match the codon usage of the host.

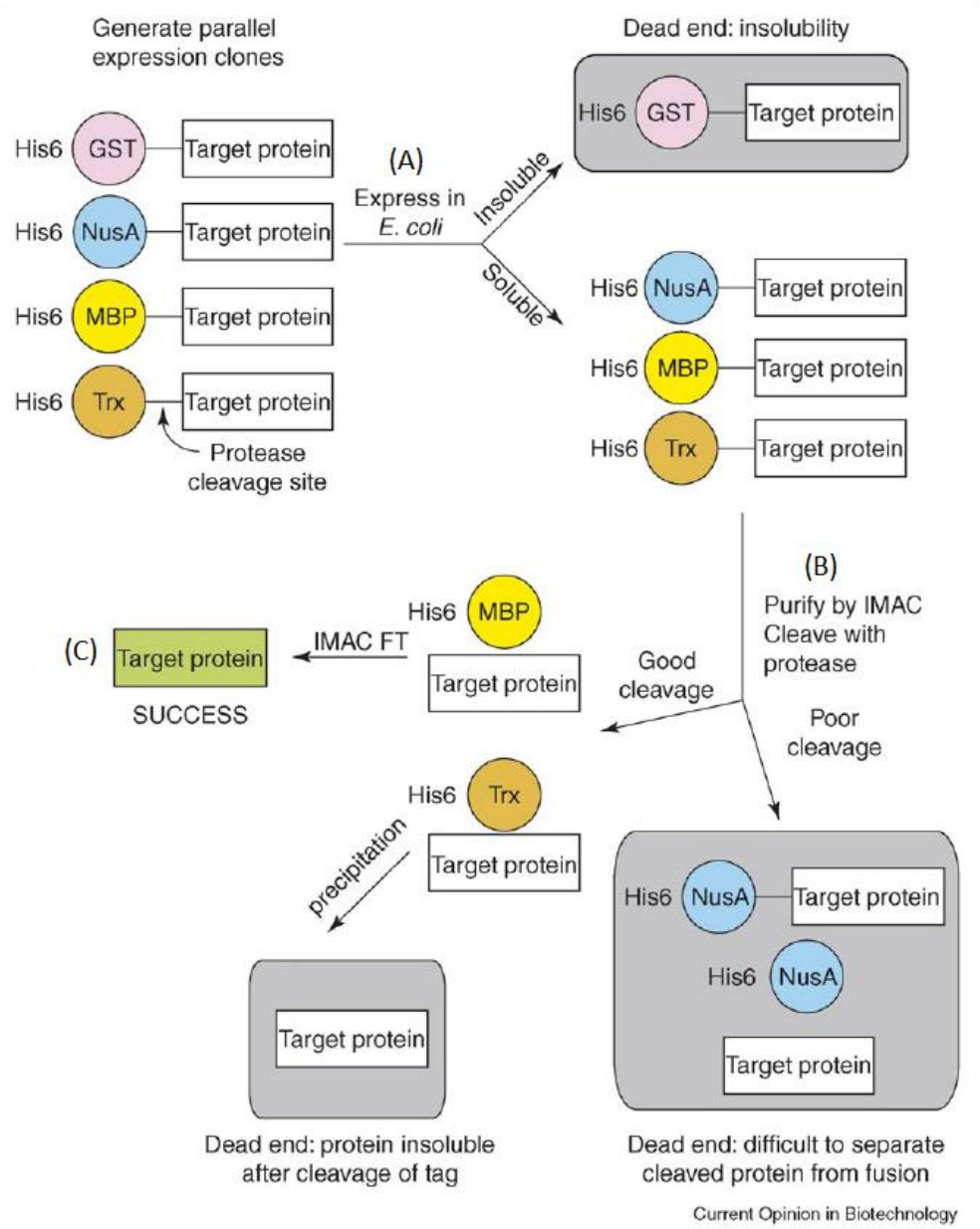

Figure 3.1.1. Enhancement of protein solubility with solubility tags. Different tags are tested for their capability to keep the protein of target soluble (A) and from good candidate tags the ones that can be removed from the protein are selected after purification (B). Finally the fusion tag can be removed by same purification method and if the target protein remains solube afterwards (C) the process has been successful (adapted from Esposito & Chatterjee 2006).

Although prokaryotic cells are less complex than eukaryotic ones when it comes to intracellular compartments and organization, targeting of heterologous proteins in *E. coli* is an important factor that has to be taken into account and with which different goals can be achieved (Cornelis 2000, Makrides 1996). Proteins can be targeted, for example, to periplasmid space, extracellular space or outer cell membrane (Figure 3.1.2). Many genes' products are involved in targeting process and understanding how

they work is important if recombinant proteins are to targeted to the right compartment. Targeting to periplasmic space offers a possibility to form cystine bonds in proteins as this compartment is oxidizing. This can be done also with cells with engineered thioredoxin pathway, but toxic proteins may have to be targeted to periplasmic space anyways. Extracellular targeting of proteins reduces the toxicity of overexpression of genes to the cells and makes it easier to collect the proteins. Display of proteins on outer cell membrane makes interactions between extracellular molecules and these proteins possible, allowing for example collection of different metals from the solution with the cells (Kuroda & Ueda 2010). Recovery of metals with surface displayed metal-binding proteins is a good example of a bioprocess where protein production and subsequent purification is not the primary goal, but where protein expression and folding play crucial roles.

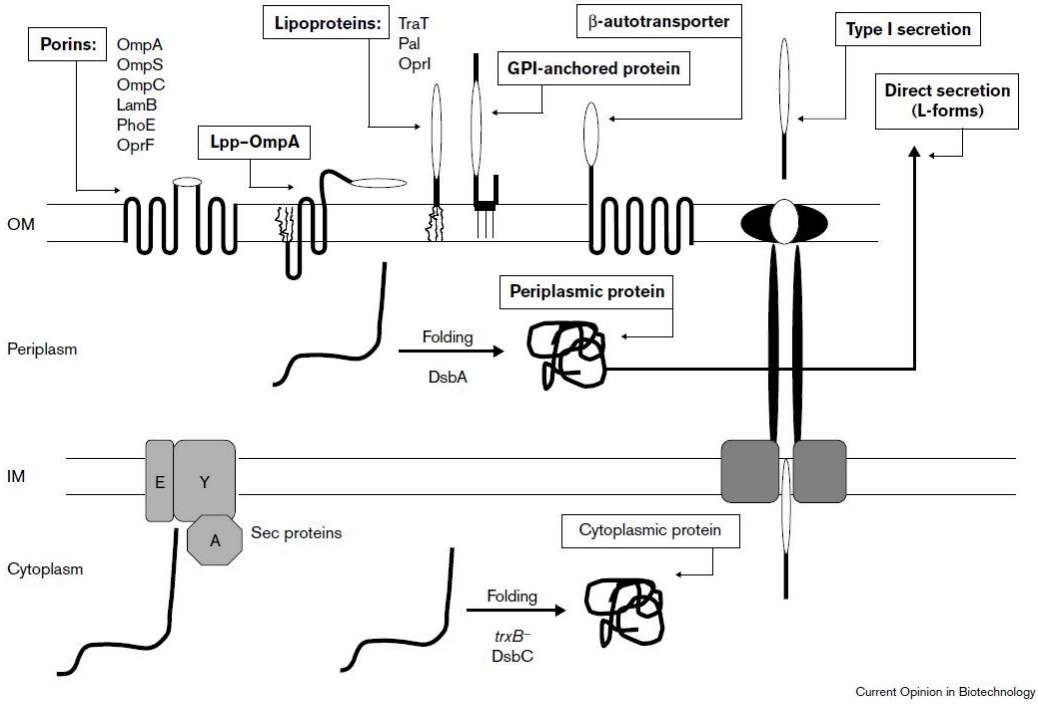

Figure 3.1.2. Different compartments of Escherichia coli to which the expressed genes' products can be targeted. The abbreviations are IM = inner membrane and OM = outer membrane. Other abbreviations are for genes whose products are assisting in targeting the proteins to each compartment (adapted from Cornelis 2000).

The yield of recombinant protein per dry cell weight is dependent on growth rate of the *E. coli* cells (Cheng et al. 2003). In the experiment, accumulation of recombinant nuclease was studied with different growth rates and it was found that the yield of this protein was highest at growth rate of 0.05 h^{-1}. However, relatively low cell densities were obtained in this study which decreases the efficiency of protein production. The increase in production of recombinant protein at lower growth rates should be compromised with increased cell densities obtained at higher growth rates if optimal protein yields are to be achieved. The recombinant protein yield is decreased by

accumulation of acetate which can interfere with DNA, RNA, lipid and protein metabolism (Eiteman & Altman 2006). The reduction of acetate formation thus is one of primary goals for improvement of recombinant protein production in aerobic bioprocesses using *E. coli*.

In the bioprocesses where product formation is due to continue in the stationary phase cells, cell death during this phase will decrease the yield. Bacterial cells like *E. coli* can undergo programmed cell death at conditions like these, where reproduction is impossible (Nyström 1998). It may be possible to enhance production of the chemical of interest in such cases by modifying the genes contributing to the cell death and genes fighting the ageing process. It was suggested that programmed cell death would be advantageous to the cell population if the cell was surrounded by lots of the cells from same species (Nyström 1998). If the programmed cell death was cell density-based, it may hamper the attempts to grow bacteria to a high cell density, especially if some nutrients became scarce during some stages of the cultivation. On the other hand, this effect may be easier to circumvent by using bacteria that are not so prone to dying at stationary phase.

3.2. Autolysis of Bacteria

In order to collect the biomolecules produced by the bacteria, they must often be first lysed. This can be achieved, for example, by mechanical disruption of the cells or by other physical or chemical means. These steps are time-consuming and increase the cost of the biomolecule production. Therefore, it would be attractive to incorporate a genetic component into the bacteria which would cause correctly timed lysis of the bacteria. This can be achieved by, for example, incorporating the genes into the bacteria which are responsible for the ending of infection cycle of bacteriophages by disrupting the host cells. Proteins called holins produced with these genes form pores into the inner membrane of the bacteria while proteins called endolysins enter the periplasmic space through these holes and start degrading the bacterial cell wall. The time of the lysis caused by these proteins is controlled by the genes encoding for them (Young 2002). Thus, it should be possible to fine-tune the timing of the autolysis controlled by these viral genes. This could allow bioengineers to exclude the cell lysing phase from the production of the biomolecules of interest, thus making the whole process economically more feasible.

Holins are proteins encoded by virus genome and are mostly expressed in late phase of infection cycle when new viruses have been assembled. They form a micron scale hole in the inner membrane of the host cell (Figure 3.2.1) which functions as a passageway for prefolded lysins to enter the periplasmic space where they can degrade the rigidity-giving cell wall (Dewey et al. 2010). Even though holins accumulate in the cytosol during the earlier phases of the infection cycle, they do not disrupt the proton motive force of the cell membrane until seconds before the lysis occurs (Gründling et al. 2001). Therefore, the presence of these proteins in cells should not cause much harm to

them before they are to be lysed. On the other hand, if molecules comprising the proton motive force are applied to a cell expressing a holin, cell lysis occurs readily if holins are present in the membrane (Young 2002).

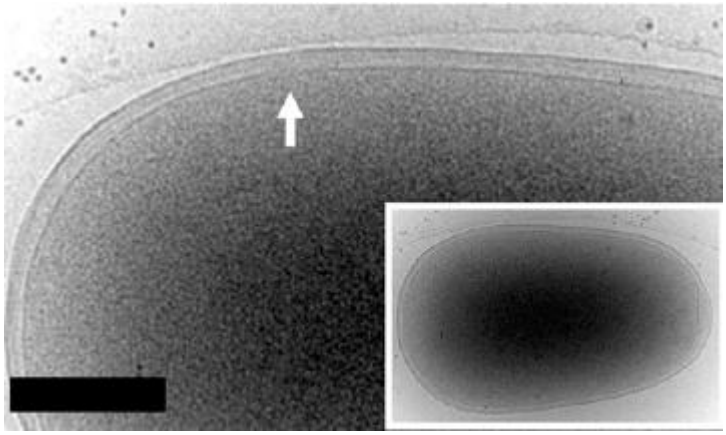

Figure 3.2.1. A lesion (white arrow) generated by holin in inner membrane of a bacterium expressing the protein (inset) (Dewey et al. 2010).

A genetic construct for lysis of the bacterial cells has recently been characterized (Pasotti et al. 2011). The construct consists of holin and lysin genes under an inducible promoter and antiholin gene under a weak constitutive promoter. The construct was shown to be able to lyse *E. coli* cells under control of N-3-oxohexanoyl-L-homoserine lactone-inducible promoter or heat-inducible promoter. However, the cassette failed to lyse all of the cells (Figure 3.2.2) most likely due to presence of lysis resistant mutants. Constitutive antiholin expression was necessary to prevent uninduced lysis of the cells caused by background expression of holin and lysin genes. Most of the fluorescent proteins produced by the cells were also released to the growth medium indicating that this approach could be used in collection of recombinant proteins accumulated in the cytosol.

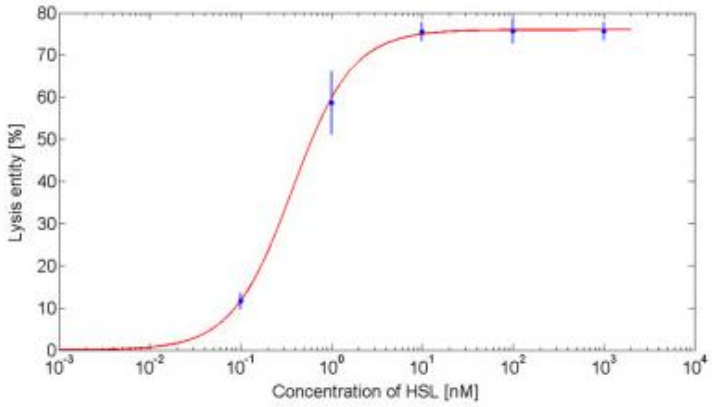

Figure 3.2.2. Lysis entity of early exponential phase E. coli cells bearing the self-destruction cassette at different inducer concentrations (Pasotti et al. 2011).

4. METABOLIC ENGINEERING

Metabolic engineering has been applied in optimization of bioprocesses to obtain various goals, including increase in product yield and elimination of side products. The main goal of metabolic engineering of bacteria for bioprocesses most commonly involves either improvement of substrate utilization or product formation (Aristidou & Penttilä 2000) as these factors have a great influence on effectiviness of the bioprocess. Although these modifications can improve drastically the yield in some cases, they can also harm the bioprocess by, for example reducing the end product yield and growth of the cells (Zhang et al. 2010). As is shown in the paper by Zhang et al. (2010), these problems can be solved by further rational modifications leading to much better yields. In this case 80 % of theoretical yield of succinate production from glycerol was achieved without introducing any foreign genes to *Escherichia coli*. However, from this example it is clear that, when modifying important metabolic routes of microorganisms, it is important to consider the effects these changes have on the main metabolic parameters of the cell, in this case on redox ratio and net ATP production. If the metabolic balance of the organism is disturbed, it is important to identify the enzymes affected by the changes. For example, in the case of *E. coli* engineered to produce ethanol, citrate synthase was found to be the enzyme whose low activity was responsible for poor performance of the strain (Underwood et al. 2002). Citrate synthase in *Escherichia coli* is inhibited by NADH, a common trait in gram-negative bacteria, and its elevated concentration was found to be the cause for reduction of growth in the bacterium. This inhibition of growth, and thus total ethanol yield, could be alleviated by molecules that can be used by the bacterium to convert NADH to its oxidized form or by using a citrate synthase insensitive to NADH concentrations.

Also when eliminating the genes responsible for growth inhibiting side product production one must consider the possibility that the production of these molecules may be of advantage to the cells in some point of the cultivation. Therefore, an alternative exists of introducing other microorganism in the culture that is capable of consuming mainly this substrate from the medium. This way it could be possible to decrease the side product concentration in the medium without affecting the metabolism of the organism producing the main product. On the other hand, it may not always be necessary to balance the metabolism of the engineered microorganism. The microorganism can be first grown to a sufficient cell density and the imbalanced fermentation can be induced then. The microorganism may not need to grow anymore at this point if the desired end product is produced.

Acetate production reduction with improved biomass yield as compared to wild type strain has been achieved in *E. coli* by removing multiple genes from its genome (Kang et al. 2009). These genes involve *Pta* and *poxB* which are involved in acetate production from pyruvate and acetyl-CoA, respectively, *iclR* which represses glyoxylate cycle, and *ptsG* which phosphorylates glucose while forming pyruvate from phosphoenol pyruvate. Activation of glyoxylate cycle allows the reduction of acetyl-CoA concentration which would otherwise cause overflow of citric acid cycle. This study highlights the possibility to compensate the harmful effects on *E. coli* metabolism by introduction of other alterations to its genome.

Cytochrome c oxidase (or complex IV) of electron transfer chain is the enzyme responsible for, in addition to oxidation of cytochrome c as its name implies, reduction of molecular oxygen to water. By modifying *E. coli* genome by removing its cytochrome c oxidases strains that are deficient in oxidative phosphorylation, and thus can produce ATP only by fermentative processes, have been engineered (Portnoy et al. 2008). After these strains were allowed to evolve using glucose in M9 medium they were shown to be able to produce mainly D-lactate or D-lactate and other fermentative acids at high yields in aerobic fermentation. Since these strains cannot use oxygen as an electron acceptor they can be used in aerobic production of fermentation products that are normally produced only under anaerobic conditions. This offers a great advantage as anoxic conditions are difficult to maintain. This experiment demonstrated clearly that by allowing the bacteria, whose metabolism has been engineered and which thus have reduced fitness under normal growth conditions, to adapt to live with these modifications can compensate for the reduced fitness. All of the modified and evolved strains showed increased lactate dehydrogenase (*ldhA*) expression during aerobic and anaerobic growth as compared to their parental wild-type strain. As a result, one of the strains also showed almost hundred-fold increase in lactate secretion rate as compared to this parental wild-type strain while its growth rate was comparable to growth rate of the wild-type *E. coli*. It was also able to convert glucose to lactate with a yield of approximately 80 %.

The use of recombinant organisms has caused some public opposition, a problem that can make commercialization of metabolically engineered *E. coli's* more difficult. This problem can be circumvented by engineering the bacteria by removing some of their genes so that foreign DNA does not have to be incorporated into their genomes. This way an *E. coli* strain that can produce ethanol from glucose and xylose has been produced (Kim et al. 2007). However, with this kind of approach, bioengineers have less possibilities to enhance the microorganisms as obviously all necessary genes for the most efficient metabolically engineered bacteria cannot be found in their genomes. Nevertheless, the above-mentioned study highlights that metabolic fluxes can be redirected by removing existing genes, a fact that should not be forgotten when producing recombinant microorganisms.

Metabolic engineering could also be used to create complex systems in which bacteria exchanged information with the fermentor's sensors. This way the cells could

respond to information received from other cells and fermentor enabling, for example, correctly timed expression of genes involved in product formation or lysis of the cells. Bacterial cells are capable of doing highly complex computations and genetic circuits that enable the cells to communicate with integrated circuits have already been constructed (Simpson et al. 2001). Thus, some of the fermentor's sensing or actuating functions could possibly be performed by the cells themselves, making the bioprocess simpler and perhaps economically more feasible. Some of the functions normally carried out by the fermentor or laboratory workers have already been replaced in some applications by engineering the cells' metabolism. For example, induction of recombinant protein production, which is normally carried out by addition of some inducer molecule, such as IPTG, has been achieved by using a native quorum sensing system of *E. coli* (Tsao et al. 2010).

4.1. Synthetic Biology

The development of genetic circuits capable of performing complex functions is often referred to as synthetic biology, which is a relatively new branch of genetic engineering. Synthetic biology is advancing at a high speed which is at least partly due to projects coordinated by BioBricks foundation where genetic circuits and the individual genes from which they consist of are modified to forms that are easy to engineer. The genetic constructs produced can be ordered from the community for free which makes them available for all researchers. All parts contain similar sets of restriction enzyme recognition sites which greatly reduces the time needed to produce new BioBrick parts (Figure 4.1.1). The list of available parts increases steadily and thus it becomes possible to use them for diverse array of applications. As more complicated and effective parts are being produced, their application in bioprocesses should become more attractive. Because of the standardization of the genetic components and the ease with which they can be used to construct new genetic systems, the researchers using them do not need to be experts of genetic engineering. This is examplified by a competition arranged for undergraduate students called International Genetic Engineering Machine competition (Vinson 2010). The constructs produced in the competition may be quite complex, for example a caffeine concentration measurement system that works in yeast (Campbell 2005), some of which may be useful in real life applications. Because the tools used in genetic engineering have become much easier to use due to development of synthetic biology and involvement of greater number of people in designing new applications, it can be said that genetic engineering is changing to become more powerful field of engineering. Engineering biology no more requires extensive knowledge about molecular biology and biochemistry thanks to new level of abstraction of genes. Therefore, it can be assumed that the constructs used in genetic engineering will become much more efficient in near future and they will become an important tool in making bioprocesses economically feasible. Thus it is of utmost importance for anyone working on bioprocesses to master the techniques used in this new field of genetic engineering.

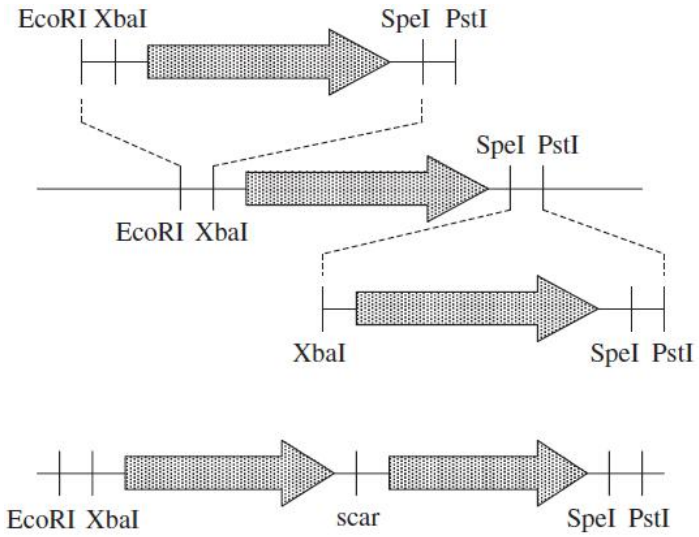

Figure 4.1.1. A schematic representation of production of BioBrick genetic construct. All BioBrick parts contain similar set of restriction endonuclease recognition sites. XbaI and SpeI produce similar overhanging ends when used cut DNA at their recognition sites allowing a part to be added to either side of another part (upper part of the figure). The produced construct (lower part of the figure) contains a mixture of these two recognition sites between individual parts that cannot be cutted with either of the enzymes (adapted from French 2009).

4.2. Synthetic Pathways: 1-Butanol Production

Even though the examples presented above have demonstrated that metabolic engineering can be used to increase the yields of bioprocesses significantly, the modification of host organism's metabolism is not always straightforward. This has proved to be the case in generation of recombinant *E. coli* strain that could be used in anaerobic 1-butanol production by transferring to it the metabolic pathway for this product's formation from a clostridial species (Atsumi et al. 2008). Thus it cannot be taken as granted that a pathway, that is responsible for production of some molecule in high amounts in its natural host organism, could be used to produce this molecule in high amounts in all heterologous hosts. In this particular case, it was found out that there was no driving force for production of 1-butanol in *E. coli* (Shen et al. 2011). Usually, when producing organic acids similar to 1-butanol, the metabolic pathway for the target molecule ends with an irreversible reaction, such as decarboxylation. Thus the authors had concluded that if some step in the metabolic pathway transferred to *E. coli* could be made irreversible, the yield could be increased sufficiently, which proved to be the case. The authors had generated an *E. coli* strain that could not produce enzymes catalyzing reactions that it normally would use for recycling NADH back to its oxidized form (Figure 4.2.1). In other words, the strain could not produce organic acids and thus could not survive under anoxic conditions. The metabolic pathway incorporated into the cells originally used two sources of reducing power but was engineered so that only NADH could be used. Therefore, in order to survive, the cells had to rely on this particular pathway. Another force driving the production of 1-butanol was the

accumulation of acetyl-CoA which was caused by deletion of enzymatic activities leading to formation of organic acids. These deletions lowered to amount of ATP produced, as formation of acetate from acetyl-CoA involves procution of one ATP molecule, and simultaneously increased the amount of starting material for 1-butanol production by increasing the amount of acetyl-CoA. The metabolic pathway producing 1-butanol was made irreversible by replacing the enzyme in the pathway that produces butyryl-CoA from crotonyl-CoA with an enzyme from *Treponema denticola* that catalyzes the same reaction but using NADH in a manner that is practically irreversible. This coupling of the driving forces to the metabolic pathway incoprorated into the host genome resulted in 1-butanol production 10 times higher than was obtained without this coupling, giving a final yield of 30 g/l (Shen et al. 2011).

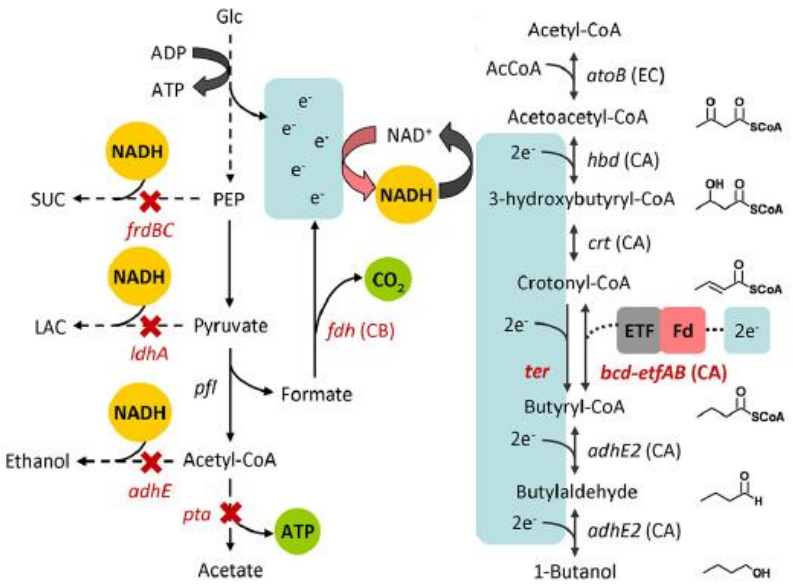

Figure 4.2.1. The modifications made to the host organism's metabolism (on left) and the 1-butanol synthesis pathway incorporated into the host organism (adapted from Shen et al. 2011).

The example presented above highlighted the importance of establishing suitable driving forces that allow more efficient product formation in bioprocesses. Furthermore, it showed that problems encountered in metabolic engineering of microorganisms used in bioprocesses can be solved by further modifying the metabolism of the host organism so that genes not directly involved in the product formation are modified or deleted. By circumventing this obstacle, a 1-butanol yield comparable to what clostridial species that naturally produce this molecule, was obtained in an organism for which bioprocess conditions have been optimized. *Escherichia coli* has advantages of being well-studied by geneticists and being easy to engineer genetically over one of the most often used microorganisms for 1-butanol production, *Clostridium acetobutylicum*. Therefore, the problems encountered in using the above-mentioned natural 1-butanol producer, for example, production of other organic acids, ethanol and acetone, can be solved by using *E. coli* in this application.

5. DISCUSSION AND CONCLUSIONS

Escherichia coli is a well studied and widely used in industry. It is used to produce many kinds of molecules for research and clinical applications, for example growth factors and interleukins. Therefore it is important to be able to use it as efficiently as possible in bioprocesses. Because of its importance to academic world and industry, *E. coli* has continued to be a popular microorganism in basic biological studies and also most of the information available on bioprocess optimization is based on cultivations of this bacterium. Since bacteria are relatively simple and easy to cultivate, they are an attractive choice of host organism in many applications where molecules are produced in large quantities. Although *E. coli* is simpler if compared to multicellular eukaryotes, its complexity should not be underestimated. *E. coli* cells are somewhat compartmentalized. For example, its DNA replication including replication of plasmid DNA is compartmentalized (Bravo et al. 2005). This complexity must be taken into account if successful protein production is to be achieved and, therefore, basic biological knowledge on this bacterium is of utmost importance for bioengineers.

Bioprocess' yield is affected by numerous factors, such as efficiency of substrate usage, cell density, cultivation conditions, efficiency of expression of genes responsible for product formation, and metabolic fluxes towards end product. All of these factors are important to consider in bioprocesses if good yields are to be achieved. None of them individually is more important than others. For example, it is useless to try to obtain high cell densities if it diminishes production of the target molecule. Rather, it is important to optimize all factors so that maximum yield and productivity can be obtained in economically feasible bioprocess.

Substrate usage should not cause problems in *E. coli* bioprocesses if only one substrate is used. Especially D-glucose, one of the most used substrates in *E. coli* bioprocesses, is consumed from the medium quite efficiently. Glucose is the monomer of starch, a widely available raw material obtained from starch crops, such as corn, wheat and cassava. E. coli is also able to utilize sucrose, carbohydrate component in sugar crops such as sugar cane and beet. However, if multiple sugars, especially C6 and C5 sugars, are to be used problems will probably arise. This is usually due to catabolite repression where more attractive substrate, such as D-glucose, represses consumption of less attractive substrates, such as D-xylose. Of course consumption of all substrates is not possible by *E. coli* as it may lack genes allowing it to consume them. These problems have been solved by means of genetic engineering in case of some substrate mixtures but if other substrate combinations are to be used, similar problems will surely arise.

Cell density achieved in bioprocesses may be limited by several factors. In case of *E. coli*, the most common of these is acetate accumulation due to overflow metabolism. Many approaches have been developed that allow *E. coli* to be grown to high densities. All of them have their pros and cons but one could mention substrate feeding limitation as one of most successful strategies. Dialysis of growth-inhibiting molecules has been used to achieve exceptionally high cell densities but it is quite wastefull in terms of lost substrate due to dialysis. It is noteworthy that high cell densities can be obtained also by just modifying growth medium to suit better the needs of the dividing cells.

It is important to be able to monitor cultivation conditions of the bioprocess. Some of the parameters can be easily measured online but others must be measured separately with other apparatuses. Aeration should be sufficient to provide the cells with enough oxygen evenly to all cells in the fermentor. The uneven distribution is also a problem in substrate feeding. Differences in oxygen and substrate distributions cause heterogeneity of living conditions in growth medium which can cause, for example, overflow metabolism that can reduce cell densities. These problems can be alleviated by efficient mixing of the growth medium. However, they are pronounced in larger fermentors where the differences between different parts of the medium can be much greater.

Product yield can be maximized by ensuring that genes responsible for product formation are expressed efficiently. In many cases this means that target proteins are produced in high quantities in biologically active form. In order to achieve this, *E. coli*'s biology and genetics must be well understood. Any foreign proteins must not be toxic to the cells and it must be ensured that the reactions they catalyze do not disrupt their metabolic balance. This is especially important in metabolic engineering applications, which have been shown to be quite promising in increasing yields in many bioprocesses. Synthetic biology, a rapidly advancing branch of genetic engineering, has been used to produce complex genetic circuits in wide variety of applications. These circuits could be used, for example, to reduce the number of functions carried out by the fermentor or its sensors or simply just to increase the yield of the bioprocess. It was also hypothesized that the processes could be made economically more feasible by lysing the cells in the end of the cultivation by introducing viral lysis genes into their genomes. If this approach was successfully used in bioprocesses, separate lysis phase of the product collection could be eliminated making the whole process simpler.

Although a lot of progress has been made in optimizing bioprocesses using *E. coli* to produce various molecules, there is still much work to be done. New problems will arise when new molecules' production in this bacterium is attempted. Bioprocesses and fermentors can still be further optimized so that the processes could be made more efficient and economically more feasible. This includes use of substrates more difficult to use in *E. coli* bioprocesses, such as lignocellulose-derived carbohydrates, which could potentially provide an inexpensive carbon source. Most importantly, new bacteriological methods and computational models need to be developed so that designing bioprocesses in future could be made easier, which would lead to harnessing *E. coli* in wider range of applications.

REFERENCES

Agarwal, L., Isar, J., Meghwanshi, G.K. & Saxena, R.K. 2006. A cost effective fermentative production of succinic acid from cane molasses and corn steep liqour by *Escherichia coli*. Journal of applied microbiology 100, pp. 1348-1354.

Aristidou, A. & Penttilä, M. 2000. Metabolic engineering applications to renewable resource utilization. Current opinion in biotechnology 11, pp. 187-198.

Atsumi, S., Cann, A.F., Connor, M.R., Shen, C.R., Smith, K.M., Brynildsen, M.P., Chou, K.J.Y., Hanai, T. & Liao, J.C. 2008. Metabolic engineering of *Escherichia coli* for 1-butanol production. Metabolic engineering 10, pp. 305-311.

Baneyx, F. 1999. Recombinant protein expression in *Escherichia coli*. Current opinion in biotechnology 10, pp. 411-421.

Baneyx, F. & Mujacic, M. 2004. Recombinant protein folding and misfolding in *Escherichia coli*. Nature biotechnology 22, pp. 1399-1408.

Biener, R., Steinkämper, A. & Hofmann, J. 2010. Calorimetric control for high cell density cultivation of a recombinant *Escherichia coli* strain. Journal of biotechnology 146, pp. 45-53.

Blount, Z.D., Borland, C.Z. & Lenski, R.E. 2008. Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*. Proceedings of the national academy of sciences of the United States of America 105, pp. 7899-7906.

Bravo, A., Serrano-Heras, G. & Salas, M. 2005. Compartmentalization of prokaryotic DNA replication. FEMS microbiology reviews 29, pp. 25-47.

von Bodman, S.B., Willey, J.M. & Diggle, S.P. 2008. Cell-cell communication in bacteria: united we stand. Journal of bacteriology 190, pp. 4377-4391.

Campbell, A.M. 2005. Meeting report: synthetic biology jamboree for undergraduates. Cell biology education 4, pp. 19-23.

Carbonell, X., Corchero, J.L., Cubarsí, R., Vila, P. & Villaverde, A. 2002. Control of *Escherichia coli* growth rate through cell density. Microbiological research 157, pp. 257-265.

Carr, E.L., Kämpfer, P., Patel, B.K.C., Gürtler, V. & Seviour, R.J. 2003. Seven novel species of *Acinetobacter* isolated from activated sludge. International journal of systematic and evolutionary microbiology 53, pp. 953-963.

Cheng, L.-C., Hor, L.-I., Wu, J.-Y. & Chen, T.-L. 2003. Effect of specific growth rate on the production of a recombinant nuclease by *Escherichia coli*. Biochemical engineering journal 14, pp. 101-107.

Choi, J.H., Keum, K.C. & Lee, S.Y. 2006. Production of recombinant proteins by high cell density culture of *Escherichia coli*. Chemical engineering science 61, pp. 876-885.

Conti, S.F. & Gettner, M.E. 1962. Electron microscopy of cellular division in *Escherichia coli*. Journal of Bacteriology 83, pp. 544-550.

Cornelis, P. 2000. Expressing genes in different *Escherichia coli* compartments. Current opinion in biotechnology 11, pp. 450-454.

Darnton, N.C., Turner, L., Rojevsky, S. & Berg, H.C. 2007. On torque and tumbling in swimming *Escherichia coli*. Journal of bacteriology 189, pp. 1756-1764.

Demain, A.L. 2000. Microbial biotechnology. Trends in biotechnology 18, pp. 26-31.

Desai, T.A. & Rao, C.V. 2010. Regulation of arabinose and xylose metabolism in *Escherichia coli*. Applied and environmental microbiology 76, pp. 1524-1532.

Dewey, J.S., Savva, C.G., White, R.L., Vitha, S., Holzenburg, A. & Young, R. 2010. Micron-scale holes terminate the phage infection cycle. Proceedings of the national academy of sciences of the United States of America 107, pp. 2219-2223.

Dien, B.S., Hespell, R.B., Wyckoff, H.A. & Bothast, R.J. 1998. Fermentation of hexose and pentose sugars using a novel ethanologenic *Escherichia coli* strain. Enzyme and microbial technology 23, pp. 366-371.

Diniz, S.C., Taciro, M.K., Gomez, J.G.C. & da Cruz Pradella, J.G. 2004. High-cell-density cultivation of *Pseudomonas putida* IPT 046 and medium-chain-length polyhydroxyalkanoate production from sugarcane carbohydrates. Applied biochemistry and biotechnology 119, pp. 51-69.

Eiteman, M.A. & Altman, E. 2006. Overcoming acetate in *Escherichia coli* recombinant protein fermentations. Trends in biotechnology 24, pp. 530-536.

Enfors, S.-O., Jahic, M., Rozkov, A., Xu, B., Hecker, M., Jürgen, B., Krüger, E., Schweder, T., Hamer, G., O'Beirne, D., Noisommit-Rizzi, N., Reuss, M., Boone, L., Hewitt, C., McFarlane, C., Nienow, A., Kovacs, T., Trägårdh, C., Fuchs, L., Revstedt, J., Friberg, P.C., Hjertager, B., Blomsten, G., Skogman, H., Hjort, S., Hoeks, F., Lin, H.-Y., Neubauer, P., van der Lans, R., Luyben, K., Vrabel, P. & Manelius, Å. 2001. Physiological responses to mixing in large scale bioreactors. Journal of biotechnology 85, pp. 175-185.

Epstein, W., Rothman-Denes, L.B. & Hesse, J. 1975. Adenosine 3´:5´-cyclic monophosphate as mediator of catabolite repression in *Escherichia coli*. Proceedings of the national academy of sciences of the United States of America 72, pp. 2300-2304.

Esposito, D. & Chatterjee, D.K. 2006. Enhancement of soluble protein expression through the use of fusion tags. Current opinion in biotechnology 17, pp. 353-358.

Fang, A., Pierson, D.L., Koenig, D.W., Mishra, S.K. & Demain, A.L. 1997. Effect of simulated microgravity and shear stress on microcin B17 production by *Escherichia coli* and on its excretion into the medium. Applied and environmental microbiology 63, pp. 4090-4092.

French, C.E. 2009. Synthetic biology and biomass conversion: a match made in heaven? Journal of the royal society interface 6, pp. 547-558.

Fuchs, C., Köster, D., Wiebusch, S., Mahr, K., Eisbrenner, G. & Märkl, H. 2002. Scale-up of dialysis fermentation for high cell density cultivation of *Escherichia coli*. Journal of biotechnology 93, pp. 243-251.

Goodell, E.W. & Schwarz, U. 1975. Sphere-rod morphogenesis of *Escherichia coli*. Journal of general microbiology 86, pp. 201-209.

Gründling, A., Manson, M.D. & Young, R. 2001. Holins kill without warning. Proceedings of the national academy of sciences of the United States of America 98, pp. 9348-9352.

Hespell, R.B., Wyckoff, H., Dien, B.S. & Bothast, R.J. 1996. Stabilization of *pet* operon plasmids and ethanol production in *Escherichia coli* strains lacking lactate dehydrogenase and pyruvate formate-lyase activities. Applied and environmental microbiology 62, pp. 4594-4597.

Inouye, S. 2010. Firefly luciferase: an adenylate-forming enzyme for multicatalytic functions. Cellular and molecular life sciences 67, pp. 387-404.

Junker, B., Lester, M., Leporati, J., Schmitt, J., Kovatch, M., Borysewicz, S., Maciejak, W., Seeley, A., Hesse, M., Connors, N., Brix, T., Creveling, E. & Salmon, P. 2006. Sustainable reduction of bioreactor contamination in an industrial fermentation pilot plant. Journal of bioscience and bioengineering 102, pp. 251-268.

Kang, Z., Geng, Y., Xia, Y.Z., Kang, J. & Qi, Q. 2009. Engineering *Escherichia coli* for an efficient aerobic fermentation platform. Journal of biotechnology 144, pp. 58-63.

Kim, J.-H., Block, D.E. & Mills, D.A. 2010. Simultaneous consumption of pentose and hexose sugars: an optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. Applied microbiology and biotechnology 88, pp. 1077-1085.

Kim, Y., Ingram, L.O. & Shanmugam, K.T. 2007. Construction of an *Escherichia coli* K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. Applied and environmental microbiology 73, pp. 1766-1771.

Kishimoto, M. & Suzuki, H. 1995. Application of an expert system to high cell density cultivation of *Escherichia coli*. Journal of fermentation and bioengineering 80, pp. 58-62.

Kleman, G.L. & Strohl, W.R. 1994. Acetate metabolism by *Escherichia coli* in high-cell-density fermentation. Applied and environmental microbiology 60, pp. 3952-3958.

Korz, D.J., Rinas, U., Hellmuth, K., Sanders, E.A. & Deckwer, W.-D. 1995. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. Journal of biotechnology 39, pp. 59-65.

Krause, M., Ukkonen, K., Haataja, T., Ruottinen, M., Glumoff, T., Neubauer, A., Neubauer, P. & Vasala, A. 2010. A novel fed-batch cultivation method provides high cell-density and improves yield of soluble recombinant proteins in shaken cultures. Microbial cell factories 9, 11.

Kruse, K. & Jülicher, F. Oscillations in cell biology. Current opinion in cell biology 17, pp. 20-26.

Kuroda, K. & Ueda, M. 2010. Engineering of microorganisms towards recovery of rare metal ions. Applied microbiology and biotechnology 87, pp. 53-60.

Lawrence, J.G. 1997. Selfish operons and speciation by gene transfer. Trends in microbiology 5, pp. 355-359.

Lawrence, J.G. & Ochman, H. 1998. Molecular archeology of the *Escherichia coli* genome. Proceedings of the national academy of sciences of the United States of America 95, pp. 9413-9417.

Lee, S.Y. 1996. High cell-density culture of *Escherichia coli*. Trends in biotechnology 14, pp. 98-105.

Lee, P.C., Lee, S.Y., Hong, S.H., Chang, H.N. & Park, S.C. 2003. Biological conversion of wood hydrolysate to succinic acid by *Anaerobiospirillum succiniciproducens*. Biotechnology letters 25, pp. 111-114.

Lin, Y.-H., Chien, W.-S., Duan, K.-J. & Chang, P.R. 2011. Effect of aeration timing and interval during very-high-gravity ethanol fermentation. Process biochemistry 46, pp. 1025-1028.

Lu, S., Eiteman, M.A. & Altman, E. 2009. Effect of CO_2 on succinate production in dual-phase *Escherichia coli* fermentations. Journal of biotechnology 143, pp. 213-223.

Lukjancenko, O., Wassenaar, T.M. & Ussery, D.W. 2010. Comparison of 61 sequenced *Escherichia coli* genomes. Microbial ecology 60, pp. 708-720.

Makrides, S.C. 1996. Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiological reviews 60, pp. 512-538.

Matte, A., Sivaraman, J., Ekiel, I., Gehring, K., Zongchao, J. & Cygler, M. 2003. Contribution of structural genomics to understanding the biology of *Escherichia coli*. Journal of bacteriology 185, pp. 3994-4002.

Madigan M.T., Martinko J.M. & Dunlap P.V. 2009. Brock Biology of Microorganisms, Twelfth Edition. Prentice Hall International, Inc.

March, J.C. & Bentley, W.E. 2004. Quorum sensing and bacterial cross-talk in biotechnology. Current opinion in biotechnology 15, pp. 495-502.

Müller, S., Ullrich, S., Lösche, A., Loffhagen, N. & Babel, W. 2000. Flow cytometric techniques to characterise physiological states of *Acinetobacter calcoaceticus*. Journal of microbiological methods 40, pp. 67-77.

Nyström, T. 1998. To be or not to be: the ultimate decision of the growth-arrested bacterial cell. FEMS microbiology reviews 21, pp. 283-290.

Ow, D.S.-W., Nissom, P.M., Philp, R., Oh, S.K.-W. & Yap, M.G.-S. 2006. Global transcriptional analysis of metabolic burden due to plasmid maintainance in *Escherichia coli* DH5α during batch fermentation. Enzyme and microbial technology 39, pp. 391-398.

Pasotti, L., Zucca, S., Lupotto, M., De Angelis, M.G.C. & Magni, P. 2011. Characterization of a synthetic bacterial self-destruction device for programmed cell death and recombinant proteins release. Journal of biological engineering 5, 8.

Peekhaus, N. & Conway, T. 1998. What's for dinner?: Entner-Doudoroff metabolism in *Escherichia coli*. Journal of bacteriology 180, pp. 3495-3502.

Portnoy, V.A, Herrgård, M.J. & Palsson, B.Ø. 2008. Aerobic fermentation of D-glucose by an evolved cytochrome oxidase-deficient *Escherichia coli* strain. Applied and environmental microbiology 74, pp. 7561-7569.

Peterson, C.N., Mandel, M.J. & Silhavy, T.J. 2005. *Escherichia coli* starvation diets: essential nutrients weigh in distinctly. Journal of bacteriology 187, pp. 7549-7553.

Ritzka, A., Sosnitza, P., Ulber, R. & Scheper, T. 1997. Fermentation monitoring and process control. Current opinion in biotechnology 8, pp. 160-164.

Shen, C.R., Lan, E.I., Dekishima, Y., Baez, A., Cho, K.M. & Liao, J.C. 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Applied and environmental microbiology 77, pp. 2905-2915.

Shiloach, J. & Fass, Rephael. 2005. Growing *E. coli* to high cell density – A historical perspective on method development. Biotechnology advances 23, pp. 345-357.

Simpson, M.L., Sayler, G.S., Fleming, J.T. & Applegate, B. 2001. Whole-cell biocomputing. Trends in Biotechnology 19, pp. 317-323.

Taneoka, A., Sakaguchi-Mikami, A., Yamazaki, T., Tsugawa, W. & Sode, K. 2009. The construction of a glucose-sensing luciferase. Biosensors and bioelectronics 25, pp. 76-81.

Thiry, M. & Cingolani, D. 2002. Optimizing scale-up fermentation processes. Trends in biotechnology 20, pp. 103-105.

Tsao, C.-Y., Hooshangi, S., Wu, H.-C., Valdes, J.J. & Bentley, W.E. 2010. Autonomous induction of recombinant proteins by minimally rewiring native quorum sensing regulon of *E. coli*. Metabolic engineering 12, pp. 291-297.

Tsien, R.Y. 1998. The green fluorescent protein. Annual review of biochemistry 67, pp. 509-544.

Underwood, S.A., Buszko, M.L., Shanmugam, K.T. & Ingram, L.O. 2002. Flux through citrate synthase limits the growth of ethanologenic *Escherichia coli* KO11 during xylose fermentation. Applied and environmental microbiology 68, pp. 1071-1081.

Vinson, V. 2010. Inventive constructions using biobricks. Science 330, p. 1629.

Winzer, K., Hardie, K.R. & Williams, P. 2002. Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch. Current opinion in microbiology 5, pp. 216-222.

Wong, I., García, M.A., Rodríguez, I., Ramos, L.B. & Olivera, V. 2003. Fermentation scale up for production of antigen K88 expressed in *Escherichia coli*. Process biochemistry 38, pp. 1295-1299.

Young, R. 2002. Bacteriophage holins: deadly diversity. Journal of molecular microbiology and biotechnology 4, pp. 21-36.

Zhang, X., Shanmugam, K.T. & Ingram, L.O. 2010. Fermentation of glycerol to succinate by metabolically engineered strains of *Escherichia coli*. Applied and environmental microbiology 76, pp. 2397-2401.