Response of Escherichia Colitonutrient Availability During Cultivationat Single Cell Level

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Preface

Summary

Industrial fermentation processes comprise the potential sources of heterogeneity since the microbial cells exhibit an intrinsic cell-to-cell variability that is both affected by and influence the fermentation process itself (reactor conditions and medium composition) over time. However, population-averaged data, assuming uniform cellular behavior in the bacterial cultures, restrict our understandings of how changing environments affect population heterogeneity or how different sub-populations contribute to the fermentation process. In this thesis, the focus was given to study and quantify single cell responses to glucose perturbations in the bioreactor using Escherichia coli as model systems.

In the **introduction chapters,** the concept of microbial population heterogeneity is briefly introduced and hereafter used as a way to study the heterogeneous fermentation process. Followed by a short experimental outline, a short description of reporter strain construction is given. The reporter system using a low copy plasmid under the regulation of the rrnB promoter or fis promoter was constructed, the expression of which may provide complementary signal in responding to nutrient availability since they have similar growth related expression pattern. Then characterization of the reporter strains was performed in **batch, fed batch and continuous cultivations** with different environmental perturbations to simulate large-scale conditions and population heterogeneity was analyzed by flow cytometry.

Oxygen and glucose oscillation, typical for large-scale cultivation, was studied in **Manuscript 1** with the two-compartment scale-down reactor consisting of a well- stirred compartment connected to a plug-flow compartment, which allows reproduction of mixing imperfections. Similar expressions of both rrnB and fis promoters were related to the cultivation mode with different glucose limitation. The sudden glucose relief elevated the ribosomal RNA synthesis as well as its transcriptional activator Fis. Heterogeneities induced by scale-down may affect protein leakage into the broth medium because of the increased membrane

permeability and thus resulted in the loss of cellular fluorescence. Such an observation was further investigated in **(Manuscript 2)** with an in-house flow cytometer and PI staining. Growth and cell permeability were monitored in real-time during the process at the single cell level and the performance of the modified on-line analysis was demonstrated by showing similar result of glucose perturbations carried out in chemostat cultivation as shown in **(Manuscript 1).** A "mean to median ratio" of recorded parameters was introduced to detect segregation in the population. At the mean time, the observed suggested that bacterial cells are robust cell factories that they could self-repair membrane integrity and thus contribute to the redistribution of subpopulations.

The study of response adaptation was extended in **Manuscript 3**. Single glucose pulse experiments were performed in aerobic glucose-limited Saccharomyces cerevisiae and Escherichia coli chemostat cultures. The physiology of S. cerevisiae and E. coli at low growth rates deserves more attention as slow-growing cells were more robust towards stress than fast- growing cells. A high GFP expression and more dynamic changes were observed in slow growing cells than fast growing cells during the glucose oscillation. Population heterogeneity was quantified by introducing new parameters to describe fluorescence distribution data.

As a complement to using reporter strains for detection of heterogeneity in the fermentation context, the use of different fluorescent stains targeting particular physiological features such as membrane integrity and metabolic activity was studied as these will provide tools that easier may be applied in an industrial setting where one maybe do not want to introduce specific reporter strains. In **manuscript 4,** characterization and application of the fluorescent dyes such as CTC, SYBR Green, P1, DiBAC4(3) and RSG was used in batch cultivation to assess cellular viability of bacterial cells as regards respiration activity and membrane potential. Cellular heterogeneity was reflected at the transcription level of the ribosornal genes and specific physiologic and metabolic characteristics of the bacteria pictured by the reporter strain and the fluorescence stains.

Sammendrag

I industrielle fermenterings processer vil der altid vaere potentiel heterogenitet, idet mikrobielle celler har en indre celle-til-celle-variabilitet, der bade pâvirkes af og pávirker selve fermenteringsprocessen (reaktorbetingelser og mediets sammens over tid. Hvis man imidlertid antager ensartet celleopførsel i en bakteriekultur, vii "polulation-averaged data" begraencse vores forstaelse for hvordan aedringer i miijøet pàvirker populations heterogeniteten og hvordan forskellige sub-populationer bidrager til fermentations processen. Stor skala fermentering bevirker, at der vii v veare en rumlig heterogenitet pa grund af utilstraekkeling omrøring, dvs der dannes gradienter af fx opløst ilt, substrater, temperatur og pH. Sâiedes er mikrobieile ceiler dyrket i store fermentorer udsat for hurtige ndringer i miljøforholdene, da de cirkulerer i hele reaktoren og heterogeniten kan have negativ effeki pa bade kvalitet og kvantitet af det dannede produkt. I denne phd afhandling er der blevet fokuseret pa effekten af varierende glukosekoncentrationer pa single cell niveau med Escherichia coli som model system.

I **introduktions** kapitleme bliver population heterogenitets begrebet introduceret og derefter benyttet til at studere mikrobiel heterogenitet i fennentations processen. Derefter følger en kort redegørelse for den

eksperirnentelle outline og en kort beskrivelse af konstruktion af reporter gener. Der blev konstrueret et reporter system ved hj afet " low copy" plasmid reguleret af en rrnB eller en fis-promotor hvis ekspression kunne give et komp1irnent signal sorn respons pa n tilg da de har samme v relaterede espressions monster. Karakterisering af reporter-stammer blev udført i **bade batch, fed-batch** og **kontinuerlige kulturer** med forskellige aendringer i miljøet for at simulere stor skala betingelser; population ndringeme blev analysered med fiowcytornetri.

Oxygen og glukose oscillation, typisk for stor skala dyrkning. hlev undcrsogt (**Manuskript 1**) i en two-compartment scale-down reaktor bestâende af en grLlndig omrort del forbundet til et " plug-flow" kammer, som giver mulighed for at undersøge mangelfuld opblanding. Lignende expressioner for bade rrnB og us promotoreme var relateret til dyrkningsbetingelser med forskellig glukose begr Det pludselige glukose puls eleverede bade ribosornal RNA-syntesen og dens transkriptional activator Fis. Heterogenitet induceret af skala-down kan pávirke protein udslip i v p grund af den forogede membran permeabilitet og resulterer sãledes i tab af ce11uI fluorescens. Denne observation blev yderligere undersøgt (**Manuskript 2**) med et in-house flowcytometer og P1 farvning. V og celle permeabilitet blev malt i real-time under processen pa single cell niveau, og resultatet af den modificerede on-line-analyse blev pavist ved at vise lignende resultater af glucose perturbationer udført i kemostat dyrkning som vist i tidligere forsøg (**Manuscript 1**). "Mean to median ratio" af registrerede parametre blev indført for at opdage segregering i populationen. At " the mean time" blev det observeret at bakterieceller er robuste cellefabrikker, at de kunne selvreparere membran integritet og dermed bidrage til en omfordeling af subpopulationer.

I **Manuscript 3**, glukose puls eksperimenter blev udført i aerobe, glukose begr Saccharomyces cerevisiae og Escherichia coil kernostat kulturer. Det ser ud til, at S. cerevisiae og E. coil ' s fysiologi ved lave v fortjener mere oprn da langsomt voksende celler er mere robuste over for stress end hurtigtvoksende. En højere GFP expression og mere dynamiske ndringer blev observeret i langsomt voksende celler end i hurtigtvoksende under glukosea

Som et supplement til at bruge reporter stammer til p af heterogenitet i forbindelse med g blev brugen af forskellige fluorescerende forbindelser benyttet til at pãvise s fysiologiske funktioner sásom rnembran integritet og metabolisk aktivitet. Det gay et v der lettere kunne anvendes i en industriel sarnmenh hvor man maske ikke onskcr at indtroducere s reporter stammer. Karakterisering og anvendelse af fluorescerende farvestoffer. som CTC, SYBR Green, P1, DiBAC4 (3) og RSG blev benvnet i batchdvrkning til at vurdere bakteriecellers levedygtighed mht. respirationsaktivitet og membranpotentiale **Manuskript 4.** Analyse af disse parametre viser. at v GFP-ekspression er konsistent med metabolisk aktivitet. idet cellerne er aktive eller inaktive med v i enten glukose medium eller i acetat adaption ' . Cel1u1 heterogenitet af de ribosomale gener korn til udtryk pa transkriptions niveau, s fysiologiske og metaboliske karakteristika af bakterierne blev visualiseret som reporter stammen og fluorescens.

List of publications

This thesis is based on the following manuscripts, which are included at the end of the thesis.

1. Han S, Delvigne F, Brognaux A, Charbon GE, Sørensen Sj. Design of growthdependent biosensors based on destabilized GFP for the detection of physiological behavior of Escherichia coil in heterogeneous bioreactors. Biotechnol. Prog. 2013.

2. Alison Brognaux. Shanshan Han, Søren J. Sorensen, Frank Delvigne. A low-cost automated flow cytometrv procedure for the characterization of microbial stress dynamics in bioreactors. In preparation

3. Anna-Lena Heins*. Shanshan Han*. Ted Johanson, Rita Lencastre Fernandes, Luisa Lundin, Gitte E Charbon. Magnus Cariquist, Krist V. Gernaey. Søren J. Sørensen, and Anna Eliasson Lantz. Shifts of population heterogeneity distributions in response to steady-state growth rate and glucose perturbations in Escherichia coil and Saccharomyces cerevisiae.* Contributed equally. In preparation

4. Luisa Lundin*. Shanshan Han*, lnês Nunes, Anna-Lena Hems, Rita Lencastre Fernandes. Gitte Charbron. Waleed Abu Al-Soud, Anna Eliasson Lantz. Krist Gernae\. Søren J. Søren Improving robustness in batch cultures by investigating heterogeiieitv at single cell evel. * Contributed equally. In preparation

CHAPTER 1:

GENERAL INTRODUCTION

Microbial populations have been traditionally thought of as large clonal groups. In fact, seemingly identical individuals display substantial heterogeneity due to the environmental sunounding [2]. Population heterogeneity has been found previously in large-scale fermentations [3,4]. Since then many efforts have been made into revealing the traditional averaged population structure. Evidence pointed that a microbial population in a fermenter is heterogeneous [3]. E. coil and S. cerevisiae have developed complex stress responding systems. Intensive studies of stress responses have focused primarily on physiology and regulation of gene expression in response to environmental changes of the key model microorganisms Escherichia coli and Saccharomyces cerevisiae [5]. Some nutrient and stress response in bioprocess will be detailed in chapter 2, but first in this introduction I will try to illustrate the adaptive response, normal stressors in bioprocess and a simple introduce of ways to visualize heterogeneity.

Nutritional and environmental factors required for growth

Cell growth (the increase in cell mass through rnacrornolecular synthesis) requires the synthesis of cellular components, which needs proper supply of sugars, N source, sulfur, phosphate, etc, to build up the cell factory. Environmental factors, such as temperature, pH, oxygen and water availability, are determining factors that affect bacterial growth. The environment can be highly heterogeneous in terms of physical and biological parameters that affect both microbial growth and development. The nutrient availability is important for understanding bacterial growth and physiology. Glucose is the most preferred carbon source for many organisms and, accordingly, changes in glucose availability often have profound consequences in many types of cell gene expressions [6]. 'Stress is any change in the genome, proteome or environment that imposes either reduced growth or survival potentials. Such changes lead to attempts by a cell to restore a pattern of metabolism that either fits it for survival or for faster growth.' [7].

Adaptive responses to stresses/ nutrient limitation

A joint response and nutrient sensing

E. coli responds to stress by reducing growth. The cell becomes smaller and rounder, accumulates storage compounds such as glycogen and polyphosphate, changes membrane composition and DNA structure and adjust the cellular activity under carbon limitation to maintain survival The tradeoff between growth and survival is the re-distribution of cellular resources to enable a higher stress resistance at the lower growth rate [9]. E. coli has different gene and protein profile under different growth conditions and behaves differently

According to the their niche surroundings in a seemingly homogenous environrnent Cell- to-cell differences, including differences in cell cycle and cell aging, contribute to the generation of phenotypic heterogeneity that exist at all the time, even if the microenvironment is constant, and only become relevant or important during environmental perturbations [10]. Stress response is usually represented by a combination of specific responses, aiming at minimizing deleterious effects (e.g. catalase during oxidative stress), or repairing damage (e.g. protein chaperones under temperature stress, SOS system induced by DNA damage) and general responses such as the RpoS-regulated response aiming to produce a more resistant cell [11]. The reaction to nutrient limitation can be seen as the transition from an active metabolic state and fast cell division at maximal growth to a maintenance metabolism and the induction of many genes whose function is to provide maximal protection against a large variety of stress conditions. The stress response is coupled with cell growth phase in E. coli [12] or cell cycle phase in yeast [13]. All in all, physiological readjustments to environmental stresses involve multilayers of genes, enzymes and proteins. The joint response adopted by bacteria involves multiple layers of genes and metabolites interaction that is integrated by cross protection through multiple signaling networks, which has been observed previously between different types of stresses: exposure to stress of one kind increases the resistance to other stresses [14].

E. coli carbon stress response utilizes mechanisms which are part of the rpoS governed general stress response, and involve several changes in cellular physiology. Carbon stress responses displayed by E. coli when growing under carbon source limitation are similar to those encountered during stationary phase or glucose limited conditions in chemostats. fed batch cultures, or in strains with limited glucose transport capabilities [15]. Still there might be stress responses that are not activated when growing yeast in the chemostat but are activated in the batch cultures [16]. In the glucose-sensing pathway the large serine/threonine protein kinase (TOR) functions as a central controller of eukaryotic cell growth that senses and integrates the signal of nutritional status, the response of gene expression, including transcription, translation and protein stability into regulation of cell growth and cell cycle progression This conserved kinase for yeast is not subject to growth-rate control at the transcription level, but is dedicated to activate translation initiation in response to nutrients [5,17].

Changes in cell size and morphology

Bacterial growth is reflected as an increase in cell size and number of cells. Nutritional stress can change bacterial morphology. Morphological plasticity is one of the bacterial survival strategies. Bacterial cell morphological alteration is related to medium composition but not the temperature, which only has effect on the growth rate Cell size is strictly regulated in response to their access to nutrients, being larger when nutrient levels are higher Cell grows lager in rich medium than in minimal medium. In E. coli chromosomal

DNA replication is not initiated until the cell reaches an appropriate size, since the cell mass (cell size)-to-DNA ratio is always constant. Fatty acid (FA) biosynthesis is argued to be involved in regulating the size of E. coli cells in response to nutrients availability in the environment 19]. It can be assumed of as a balance between growth and cell division. Opposite changes in cell size were observed during phosphate and sulfate batch limitations Entry into stationary phase E. coli cells become smaller, develop a spherical or egg shaped rather than a rod-shaped morphology and preserve a tough membrane Morphogene bolA regulated by sigma factor S contributes to the decrease of 'surface to volume' ratio and modulation of outer membrane properties 12]. By contrast, many gram-positive bacteria produce dormant spores in response to starvation In the budding yeast Saccharoinyces cerevisiae nutrient limitation induces morphological changes that depend on the PKA pathway and the filamentous growth Kssl MAPK pathway in either diploids or haploids. The protein kinase RIM 15 functions in the integration of the nutrient signals. particular glucose depletion, into these pathways and thus mediates colony morphology Low levels of carbon combined with abundant nitrogen trigger colony morphology response in budding yeast which refers to the formation of biolilm. multicellular stalk-like structures, etc.

Changes in membrane, DNA structure, protein production and protein composition Membrane permeability, DNA integrity and protein folding are three classes of cell structure to be valued on the magnitude of stress and are central to bacterial urvival In Gram-negative bacteria such as Escherichia coli. the envelope comprises three layers. an inner membrane, a periplasmic space that contains the cell wall, and an outer membrane. Being in contact with the external medium, the envelope is the initial target or boundary 0! physical (e.g., hyperthermia, osmolality), chemical (e.g.. ethanol. pH. detergent) or biological (e.g., adhesion, infection) stresses that may alter envelope components, thus inducing an extra cytoplasmic stress response The response to most stress factors relies at least partly on the membrane integrity. An intact and robust membrane is essential to keep cytoplasmic p11 rather constant: that is how a bacterial cell physiology is tightly controlled and how well cell could cope with external stimuli Confronting

With acetate stress, genes belonging to or independent of RpoS family provide a joint protection to maintain a near neutral pH in E. coli, for instance, cyclopropane fatty acid synthase (coded by cia gene) helps to decrease the permeability of the membrane to protons Although overall protein synthesis rate is reduced to 20% in the first hours of starvation, bacteria synthesize new survival proteins characteristic to conditions of carbon, nitrogen or phos starvation 30]. Transition from glucose-excess to glucose-limited growth conditions resulted in enhanced membrane permeability of glucose in short-term and mutations of three loci (mic, mglD/O and malT) to increase glucose affinity in long-term physiological adaptation More recently, some small (low molecular weight) neglected stress response proteins are surprisingly identified under different growth conditions andl or after exposure to stress, which might count for the inner membrane permeability Outer membrane structure and protein composition can be altered by medium composition, glucose- feeding rate in batch, continuous or fed-batch cultivations 34]. In E. coli the synthesis of the major outer membrane proteins OmpC, OmpF and OmpA will be altered by different nutritional conditions Protein leakage seems to be a process related phenomena, it was found to be as twice as high in chemostat cultivation compared to the constant leakage level in fed-batch 36]. Some subpopulations of starving bacteria tends to take up foreign DNA or have an increased mutation rate, thus altering their genomes

Envelope stress is triggered by the toxic accumulation of periplasmic misfolded proteins (LamB) in the envelope under stressors like nutrient a'cailahility and heavy metals. In F. coli several extracytoplasmic pathways are activated in response to envelope stress to protect cells, including the yE pathway (responds to misfolded outer-membrane proteins), the Cpx pathway (monitors cell-surface-protein assembly), the Psp system (scnses dissipation of the proton motive force) and the Bae pathway (responds to antimicrobial compounds). Release of outer membrane vesicles is addressed as a new and independent envelope stress response{38].

Changes in substrate utilization

Substrates in a rich medium (LB) were used sequentially under optimal growth and then turned over to simultaneous utilization when substra limitation occurs [Similar utilization of fructose, mannose, maltose and ribose in combination with glucose is found in the growth - limiting chemostat cells This uptake style through phosphotransferase sugar uptake system (PTS) is independent of dilution rate. Other sugars (e.g. glycerol, galactose, lactose) through the transcription factor pathway require a lag before being used. As undesirable by-product, growth upon gluconeogenic carbon - acetate showed a rather distinct physiology through other unclear sensing routes. The shifts of acetate growing to glucose or broth bring up nutrient specific growth kinetics With the presence of either glucose or acetate, the cells adapt to each of these carbon sources; in the presence of both carbons, glucose will be preferably completed first then the cells turn to acetate Enhanced tolerance to oxidative stress and thernio is thought to be a positive effect of acetate, the induced genes of acetate belong to the rpoS family and some of them are acid protection genes [28].

Changes in metabolism and gene expression: induced/repressed

Metabolism is the totality of bacteria's chemical processes to maintain life, including reactions in glycolysis, tricarboxylic acid cycle (TCA), oxidative phosphorylation, ATP biosynthesis and amino acid metabolism. The study of metabolism in different physiological states of cell provides useful information in understanding specific metabolic pathways, or simply provides a metabolite concentration that could be related to any gene transcription. When integrated with transcriptomics and proteomics analysis, the physiological state of the cell can be fully understood. And based on the integrated result modifications in gene could be a tool for improving strain performance. especially for those strains grown under certain stimuli in bioprocess.

Shiftinn energy metabolism appears to be an important strategy in cell survival and stress responses. During starvation or under exposure to stress many bacteria experience a significant turnover (metabolic breakdown, rearrangement and re-synthesis) of protein{45. 46] and RNA (carbon and energy source). There are some other secondary metabolites with unknown function in the growth and development of the bacterial cells found in the marine environment Stimulus- response or pulse eNperiments are a typical way of metabolic investigation. By applying a substrate pulse to a substrate-limited, steady state culture. cellular metabolism is shifted away from its regular metabolic steady state. Variation in the response of the different organisms to external rimuli is reflected in secreted ethanol in S. cei'evisiae or increased storage malenai in P. c/irlsogelium, but not in E coli

Comprehensive profiling of a large amount of metabolites needs a high sampling frequency for E. coli. since the new steady state is quickly achieved in a short time period of 40-60s [48]. Pathway fluxes of

E, coli MG1655 have been verified for stress of carboxylic acids using 1 labeling methods transcription data of heat shock, osmotic shock, oxidative stress genes and TCA cycle genes from fed batch cultures has been captured with genorne scale array technique at different specific growth rates Energy conservation is a general strategy at both transcription and metabolite level, whereas, metabolite response reacts more specifically than response at transcriptase level under five conditions of cold. heat. oxidati stress, lactose diauxie and stationary phase.

Carbon and nitrogen starvation share similar induced proteins and genes with more genes induced under carbon limitation RpoS and (p)ppGpp are mostly studied as major components of the general and specific response that regulate some key cellular processes ensuring global control upon perturbation. It is interesting to classify each physiological profile under different conditions since they are quite different and nutrient specific, e.g. different densities' population has non- identical rpoS expression and membrane composition at a dilution of 0.3 h under a glucose range of 0.005-0.12% {54]; altered growth rates correlate a particular pattern of gene expression, enzyme production and mutation 53]; RpoS synthesis is unstable during phosphorus and nitrogen limitation, but becomes stable during carbon limitation; central metabolic pathway is shut down in carbon starvation but still functioning in phosphate limitation induction in rpoS transcription is inversely correlated with the decrease of the specific growth rate cells stop growing immediately in carbon and nitrogen limited assays but slowly grow in phosphate-lacking cnltures Three classes of genes encoding starvation proteins have been defined. These are the csl genes, controlled only by carbon starvation and requiring cyclic AMP/CRP; the csi genes- also induced by carbon starvation alone but independent of cAMP; and the pex genes, induced by nitrogen, or phosphorus starvation other than carbon and cAMP/CRP independent Once any damage posed on the membrane there are five signaling pathways of Bae, Cpx. Psp. Rcs, and o existing in E. coli that start simultaneously to detect stimulations and allocate cellular resources to recover from the damage With some overlaps, each path av functions distinctively. All these changes imply that cells must undergo structural changes to live on the edge of survival. A summary lists of E. coli genes that response to nutrient limitation, environmental stress, or those genes indicating the physiological tatus of the bacteria were presented by Schweder for instance. n is required to enhance its acid tolerance when confronted with pH stress in a fermentation process.

Microbial heterogeneity

The types of heterogeneity within a microbial population can be divided into four general classes: genetic level, biochemical level, physiological level and behavioral level Biochemical or behavioral differences might ultimately be traced back to a genetic basis; physiological heterogeneity. which may be driven by forces external to the cell (e.g., nutrient limitation or the presence of antibiotics), could be viewed in terms of the organism's genetic potential to respond to these forces. Genetic heterogeneity is addressed through disturbance on genetic material. Biochemical or metabolic heterogeneity is characterized by individual cellular differences in macromolecular composition or activity and may stern from diversities in physiological processes and genetic resources.

Physiological and behavior diversity: VBNC, persister, chemotaxis behavior, phenotypic modification, etc.

Sources of physiological variation in bacteria include differences in cell volume, cell shape, population density, nucleoid morphology and environmental characteristics. Assessment of physiological state of individual cells is very useful in monitoring bacterial populations in biotechnological process. The changes of bacterial cell physiological state during cell cycle and life cycle as well as interaction of cells with inhomogeneous microenvironment result in the formation of a physiologically heterogeneous cell population 1].

Behavioral heterogeneity is an observation of individual cellular responses to chernotactic or phototactic stimuli and measurement of swimming speed or direction, which is termed as cheinotaxis It is measured through direct observation of cellular responses to various stinmli. This proton gradient-dependent chernotaxis system in E. coli is reported to be affected in glucose limited fed-batch cultivation, since majority of chemotaxis genes were transcribed from active to less active in order to save energy for other cellular activities (;enetic diversit (genetic arrangement, epigenetic modification, mutations, etc) Heterogeneity could also result from changes or rearrangements at the DNA sequence level, from diverse cell cycle phases and age distributions. Mutations occurred during storage make stored archival cultures of bacteria becoming heterogeneous, which is likely to contribute to strain variation in stocks of e.g, K-12 strains in regards of its physiology. cnctics and gene regulation Epigenetic differentiation occurs stochastically but can also be triggered by external stress or changes. with a higher occurrence frequency than mutations leading to difIèrential gene expression and add complexity in an identical population. The presence. absence and the copy number of transposable genetic elements (transj-osons insertionIS elements) can diversify population genetic structure, as well as inversion. translocations. etc. Those genetic arrangements create a better adaption capability to the environmental conditions.

Biochemical or metabolic heterogeneity

Bacteria metabolic performance is largely depend on their growth rate, which is in turn controlled by their genetic material and physiological state 67]. Cellular differences in macromolecular composition is also contributes to biochemical heterogeneity. Metabolic heterogeneity is measured by substrate uptake or metabolic flux within cultures and is evidenced in many glucose pulse studies 691.

Heterogeneity inside the fermenter

A typical microbial population in a bioreactor is heterogeneous in many aspects at single- cell level 61]. Heterogeneity, to some extent, is a beneficial solution for the robustness of the fermentation process with quick adaptation to new conditions thus boosts population level fitness [3]. Significant gradients of e.g. dissolved oxygen, substrates, and pH are typically observed in many industrial scale fermentation processes Cells fluctuating in large volume reactors therefore experience significant micro-environmental changes while mixing inside the reactor, especially in aerobic fermentations Consequently, diversities in gene expression and protein translation generate the presence of subpopulations. Local microenvironments that induce dynamic cellular genetic, metabolic and physiological responses have been identified as the key driver for the development of heterogeneity in the bioprocess The resulted residence time and the relevant various types of cell responses are expected to be more distinct from each other in the bioprocess based on the sizes of the microenvironments, which in turn are responsible for causing the development of heterogeneity in a pseudo homo population The gradient fomiation effect is further magnified by reactor

enlargement as a result of deficient mixing. which leads to zones with diverse external conditions Therefore the larger reactor is the greater possibility of finding heterogeneity (Figure 1.1, A). Population fragmentation is quite bad in a fed-batch process since a highly concentrated substrate is fed through a narrow zone to avoid a significant increase in volume. The severe glucose gradients generated by improper mixing in high density fermenter were shown to be responsible for inducing four stress sensitive genes and affecting heterologous protein production, product yields and cell viability Changes in lucose availability often have profound consequences in many types of cell gene xpiessions (as simplified in Figure 1.1. B which is supported by a recent study of s. of yeast to the period of nutrient excess engendered by a glucose or ammonium : (single pulse) Although responses have been studied, not much is known about influence of such inhomogeneity on the process performance Understanding the nics of heterogeneity in bioprocesses is considered to be a valuable tool for bioprocess ' since population heterogeneity has a significant impact on the product :ncy and quality On the other hand, growing cells continuously alter their diate environment during their lifespan 16], thus, those cells with ability to adapt
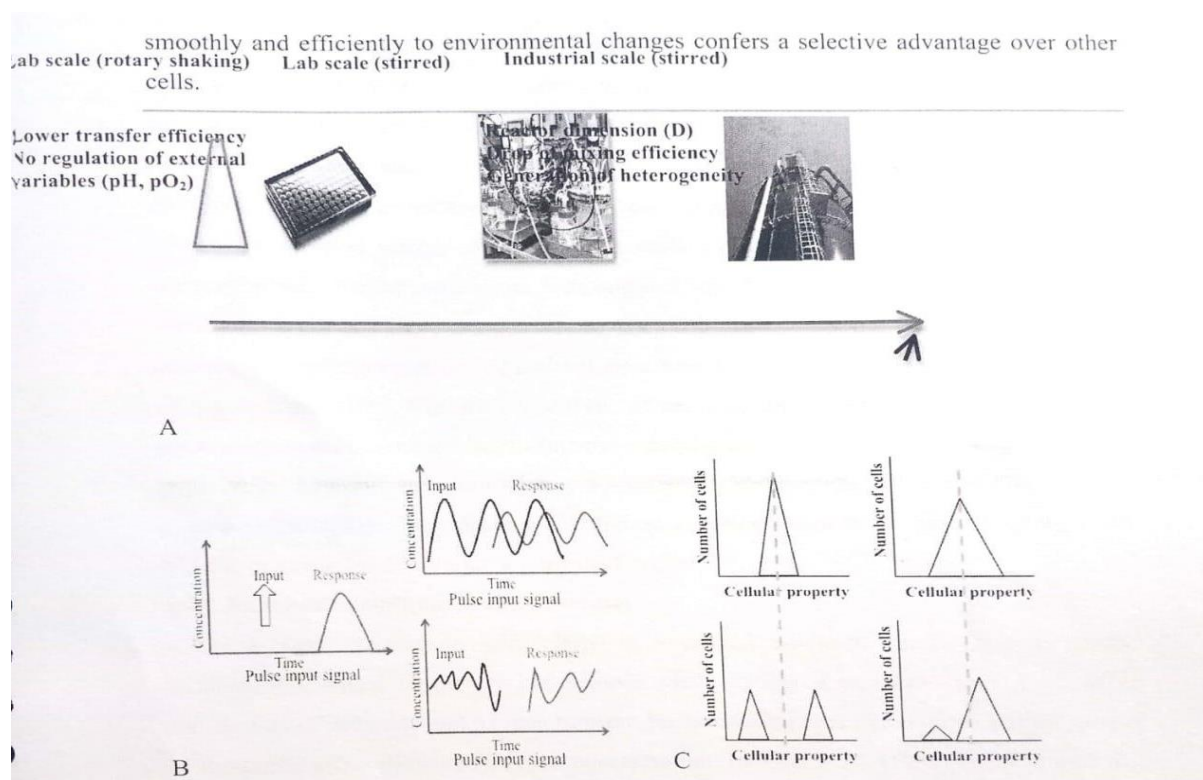
Figure 1.1 Scale up bioprocess (the generation of spatial heterogeneity, A). basic set up of perturbation studies (stimulus-response, B. adapted from Delvigne Ct cii. 2009). and potential distribution of the cellular properties(C). (Adapted from Dhar et cii 2007

Osmotic stress in the fermenter

Osmotic stress in industrial fermentations mostly is a direct consequence of product accumulation. it affects bacterial growth behavior and metabolic production in many bioprocess Hyperosmotic stress is a situation when the solute concentration inside the cell is lower than that of its environment, and vice \ersa. One of the strategies confronting higher osmotic pressure is to utilize organic intracellular solutes and/ or maintain ion concentration gradients across membrane. The immediate response of E. coli to hyperosmotic shock with NaC in the batch fermentation is a fast taking up of K to increase the internal osmolality with a concomitant intracellular increase of the glutamate and then in the subsequent phase charged ions are

exchanged by neutral solutes like proline, trehalose, glycine, betaine, etc Meghna Rajvanshi, et al. showed that at hypoosmic condition, trehalose and glutamate are the main compatible solutes for Corynebacteriurn glutarnicum but at higher osmolalities proline plays a dominant role Glutamate is shown to be important in osmotic response after sudden increase in the external osmolality. Trehalose and proline are the main protecting agent in the long-term adaptation. When it moves to continuous culture, the widely used Corynebacterium glutamicum for the industrial synthesis of glutamate is confronted with osmotic stress better at higher specific growth rates than lower because of a higher energy efficiency by a rapid changing of the osmolite Frequent encounters with fluctuations in the osmolality, E. coli has developed efficient methods to cope with hypo- and hyperosmotic stress. The osmotically sensitive gene proU (uptake of protectants of glycine betaine and proline) gives a detectable response within 15 seconds after an osmotic shock The mRNA analysis of the proU expression shows that the osmotic pressure decreases in the fed- batch cultivation despite a high cell density and accumulation of byproducts. since substrate limitation in fed-batch with feeding nutrient allows elimination of osmotic effects Time dependent different adaption behavior to osmotic stress under aerobic or anaerobic conditions is published in[

1. 4.2Anaerobic condition in the fermenter

The oxygen transfer in particularly is a critical parameter in large-scale aerobic fermentation, which frequently has a lower yield compared to small scale. The uneven distribution of substrate and oxygen transfer, the measuring port of the probe and the quality of the probe affect dissolved oxygen concentration. Larsson et al. (1996) demonstrated that oxygen gradients vary with stirrer speed, with the time of cultivation and with the organism type and process Cells passing through the oxygen-limited zones sense and respond to oxygen depletion, leading to the activation of anaerobic metabolism. Cells are more senSItive to osmic pressure when grown under anaerobic rather than aerobic condition due to a less activation of antiporter for Na extrusion in E. coli 1.4.3Acidity condition in the fermenter

The pH gradient can lead to a reduction of cell viability. Protection provided for adapting to acid stress (pH 5.5-4.5) is controlled by a mechanism known as the acid tolerance response (ATR). Aerated F. coli cultures respond to pH changes by selective expression of numerous envelope and periplasmic stress proteins, redox modulators and catabolic enzymes. The modulation of catabolism in complex medium such as Luria-Broth medium at low pH is modulated to pathways to produce various fermentation acids. Lactate is preferable produced instead of acetate and formate, with thd latter more detrimental to cells. The pH gradient may drive the fermentation acids back into the cells and the released proton after dissociation acidities the intracellular pH When encounter anaerobic condition, the excretion of weak acid (organic acid) from cells is increased and some other pathways for lighting the stress effect is available Inorganic acid or organic acid both disnipts some key biochemical processes through intracellular acidification. Furthermore, levels of phospholipids that contain membrane- stabilizing cyclopropane fatty acid [and accumulated rpoS- controlled acid shock proteins will be elevated in front of acid stress. Acid response of E. coli K12 in log phase involves different pathways when rich or minimal medium is used. The rpoS dependent oxidative system (AR1) and two distinct fermentative acid resistance systems of glutamate decarboxylase (AR2) and arginine decarboxylase (AR3) are involved in the rich medium defense But in another research study it is reported

that only AR2 system is activated in minimal medium and none of the three AR systems are activated in exponentially grown cells in rich medium

1.4.4 Oxygen gradient and substrate gradient in the fermenter

Oxygen availability is very important for E. coli growth with respect to acetate production and product yield. Oxygen gradients are frequently found in large scale bioreactors due to transport limitations when the oxygen consumption rate exceeds the oxygen transport rate. which is a situation particularly obvious in fed-batch cultivations since a highly concentrated substrate is fed through a narrow zone to avoid a significant increase in volume. E. coli cells are able to recognize gradients in a bioreactor within seconds as exposure to transient oxygen limitations, even a few seconds (13 see). is enough to induce genes of anaerobic pathways and divert essential carbon skeletons to undesirable by-products Accumulation of acetate and formate was detected after only 2s exposure to the glucose-rich (aerobic) region in the plug flow reactor

The nutrient inside the fermenter is not equally distributed. The high concentrated feeding has shown to generate substrate gradients due to repeated stiiring that creates nutrient starvation zones and oxygen limitation. More than 10.000 fold concentration difference between the feed solution and the bioreactor broth in large-scale reactor generates varied gradients depending on the location of the feed 4]. Glucose gradients generated by improper mixing in a E. coli fed— batch fermentation at top. middle aiid bottom le ek in a 22 m fermenter affect heterologous protein production. product yields and cell viabilit induce stress responses of four stress sensitive genes and bacterial cells differentiate even morphologically near the feeding point

Ways to visualize heterogeneity

Ordinary bioprocess monitoring often relies on population-based measurements of cell viability and yield production, which is unable to reflect the heterogeneity among individual cells within a population. A number of methods have been applied to discovery and monitor the cellular mechanisms and regulatory circuits on the single-cell level.

Molecular characterization of genetic variability

The most common methods used for genetic fingerprinting are the gold standard' method of pulsed-field gel electrophoresis (PFGE), two PCR-based techniques of repetitive extragenic palindromic (REP) and the enterobacterial repetitive intergenic consensus (ERIC), simplified amplified fragment length polymorphism (AFLP). suppressive subtractive hybridization (SSH), etc. The detection of specific mRNA in individual cells may provide more in situ information. RT-PCR was used to specific gene induction (groEL mRNA per cell) and general growth activity (t. per cell) The number of targeted and per cell was first announced in Kim Holmstrøm's work

Green fluorescent protein and related reporter proteins

The capacity of being a reporter protein has been revalued from the point of accuracy and reproducibility. Reporter proteins such as luminescence and fluorescence have served as in a variety of biological studies. Standard reporter proteins have very slow clearance rare and thus are quite stable, for instance. chioramphenicol acetyl transferase (CAT). beta galactosidase (and enhanced green fluorescent protein (EGFP) have protein half-lives of —50, 30 and 24 h. respectively Unlike several other commonly used reporters. the application of GFP is quite simple and does not require additional cofactors or external

substrates. The GFP protein has been modified to produce fluorescent proteins with a variety of colors including blue. cyan. yellow, and red, making it flexible to use in biological studies [89]

GFP and its derivatives are widely used in biological studies since it does not interfere with cell growth however, release of GFP to the supernatant is regarded as a tracer of membrane integrity and cell death The evaluation of cell damage using this way could not be consistent all the time, since the release of cellular contents is a size dependent process. As the high degree of stability of the wild type GEP protein (the fluorescent signal can last for over 24 hours I]). non— active. non—viable or dormant bacteria cells containing stable GFP would continue to be fluorescent or still retain GFP fluorescence in the absence of promoter gene expression and the fluorescence will extend over many bacteria generations, it might pose a problem in experiments where transient or dynamic gene expression is concerned. Therefore. the use of a destabilized reporter system is preferred. Driven by this purpose. Andersen c al. made destabilized variants of

GFP by adding a C- terminal oligopeptide extension that targets GFP for degradation through an ssrrA –mediated degradation systern SsrA. also referred as lOSa R\A or tmRNA, tags are recognized by SmpB (small protein B) and are targeted for proteolytic degradation The half-life of tagged proteins in E. coli is dependent on the last three amino acids of the C-ten tail Addition of an ASV-. AAV- and LVA -tag reduces the half-life of GFPmu from 24 hours to 110 mm, 60 mm and 40 mm, respectively It has been experimentally identified that the half-life of these GFPs is different between E. coli and P. putida and has more weight than mRNA half-lives in interpreting the gene expression dynamics Still, the mRNA stability of reporter protein contributes to the degradation of the reporter protein and might interfere with sensitive and fast detection of changes in gene expression. Improvement has been made in constructing both reporter protein and its R1' with short half-life in double destabilized reporters The feature of rapid degradation of these fluorescent proteins makes it very useful in in situ studies of temporal gene expression. The unstable GFPs have found multiple applications, such as dynamic monitoring of bacteria growth rate But, it is a nuisance that the expression of unstable GFPs with a weak promoter cannot be detectable due to analytical limits. And its fluorescent property in different strains is not the same Likewise. luciferase is intrinsically less stable than GFP and its half-life can be further i-educed by similar tag fusion. Short half-life variants of LuxA and LuxB protein from Photorhabc/us /u,ninc'scem were constructed into E. coli by fusing an 11-amino acid C- terminal tag that is the target of endogenous tail-specific proteases Another, the application of using the destabilized GFP (yEGFP3 - CIfl2PEST) with estimated half-life of 30 mm or so has been extended to yeast to detect cell cycle-dependent gene expression [To date, there are over 10 different color variants commercially available including blue, cvan, yellow and red.

GFP is the most well kiiown fluorescent protein, however, one need to be aware that GFP signal may be interfered From sample handling, at least, population quality differs [The influence of delay in measuring is not '.crv clear and is rather personal. I;xcept a single fluorescence gene, dual fluorescence proteins are introduced into the same plasmid to study cell—to—cell variabilit\ in protein expression [.•\ ne lv made flavin mononucleotide (FM\)—hased oxygen-independent fluorescent protein is alternatively [tested in plasmid transfer feasibility together with a red fluorescent protein under both aerobic and anaerobic conditions \evertheless. it has a potential in oxygen availability studies since GFP and other protein require oxygen in their maturation process. 3-galactosidase, which is encoded by the bacterial gene IucZ. can be used

as a reporter gene (e.g. the best known blue white selection) but not an ideal choice in time scales study. As one of the first reporter gene, B- galactosidase assay is widely used in food industry{ 104]. in distinguishing subpopulations [and protein leakage study in fed-batch cultivation

Fluorescent dyes and stains

Staining techniques allow the determination of individual cell viability, activity, surface properties, etc. which are the most relevant physiological parameters to be assessed. The study of the different physiological state of a cell has been redefined by the application of flow cytornetry in the fennentation process and a range of stains is available for studies of single cell characteristics by flow cytometry. The interpretation of bioprocess data often fails to reflect the actual state of individual cells within a population since, as reported by several researchers, cells are not just alive or dead, but have a range of intermitted states (Figure l.2) 108]. Therefore. more and more applications tend to rely on using the combination of fluorescent dyes and by staining with appropriate fluorochrome the physiological state of both subpopulations and individual cells can be quantified and distinguished between vital (i.e., active and viable) and compromised (or dead) cells
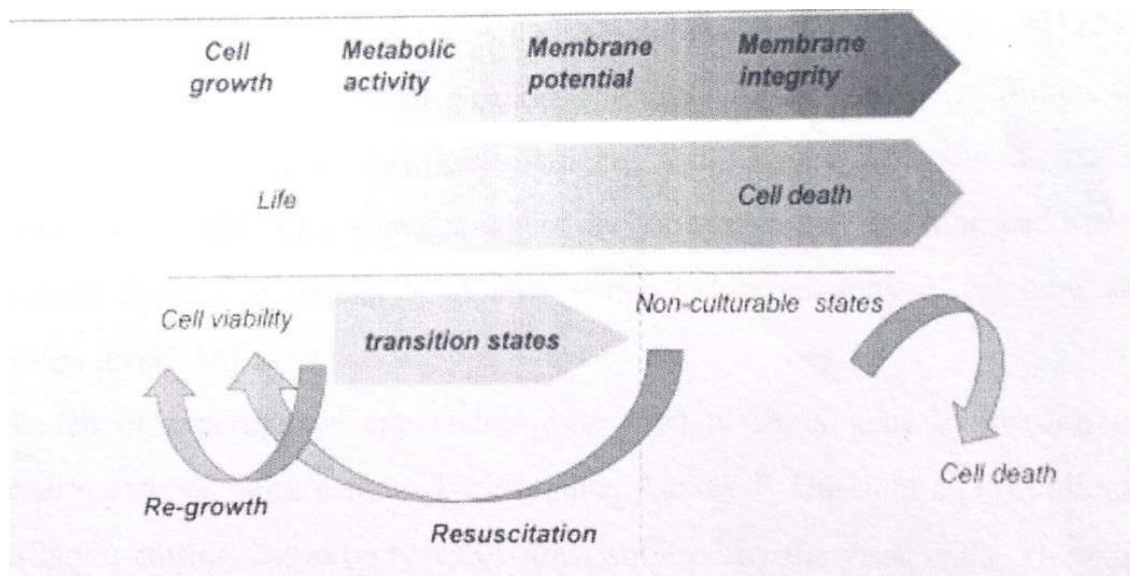
figure 1.2 LIfe and death spectrum: different levels of cell viability and vitality in the physiogical assessment [107]

Single cell and global analysis

(Hinpiex physiological behavior emerges from complex interactions between transcription level nene expressions. metabolism and enzymes. Ordinary bioprocess monitoring often dies on population-based measurements of' cell viability and yield production, hich is ui; to reflect the heterogeneity among individual cells within a population It is H:cresting to study the potential distribution of the cellular properties (Figure 1.1, C) or transient cellular responses that overlooked by measurements on population level. The ability of accurate measuremcnt has rapidly accelerated over time with the integration of omics and singic cell analysis. Properties of single cells within a population can be quantified by flow cytometry without averaging any signal intensity, for instance, unveiling cell-to-cell differences and thereby the heterogeneity of the population Application of fluorescent stains for assessment of viability and physiological state of individual cells together with flow cytometry bring great potential that cells of interest can be sorted Out and cultured afterwards. omics" based technologies have enabled the direct measurement of internal variables

within the cell and contribute to expand our understanding of stress responses in face of environmental changes. Global relative changes in gene expression are thus comparable. Using DNA arrays, the relative gene transcription can be rapidly quantified and thus overall physiological response of the cell could be captured 11-113]. Population fitness in biofilm formation has been contributed to the arcA gene in a DNA array study 12] and is visualized in Philip S and coworker's work 14]. More yeast genes on the chromosome were induced (and to a higher levels) in the carbon-limited chemostat than in nitrogen-limited chemostat as proved by using hybridization array method

Experimental tools

Stress responses at gene expression level are transient, leading to ne steady state levels similar to the unstressed cells even in the continued presence of the stress 15]. External perturbations can prevent optimal enzyme activities, disrupt metabolic fluxes, destabilize cellular structures, perturb chemical gradients. etc.. leading to overall instability Depending on the duration and magnitude of the perturbation the duration and the magnitude of transient changes in cellular RNA level varied before adapting to the new steady state expression level 16].

A bunch of experimental approaches were used to study gene expression of cells in response to external perturbations. For example. Audrey P. Gasch et al 16] allocated genes into different clusters based on types of stress suffered by the yeast cells. The authors were able to sct a diverse group of stress factors (temperature shocks, hydrogen peroxide, hvper and hypo-osrnotic shock, amino acid starvation and so on) and describe stress response at genomic level. A similar experimental design was undertaken by Harald Weber 17] to identify genes whose expression were RpoS dependent in the E. coli genome: up to 10% of E. (oil genes are Linder the control of RpoS. The global gene expression analyses under defined cultivation conditions enable the identification of genes. the expression of which is relevant and specific for processes evaluations 16, 115]. Schweder recently summarized E. coli genes that respond to nutrient limitation, environmental stress, or those genes indicating the physiological status of the bacteria.

General introduction

Another way of looking into stress response is to perturb the steady state system with pulses of substrates or sudden shifts of oxygen or temperature. Sirichai Sunya 18] compared dynamic cell responses under a single glucose pulse of different intensities (0.08, 0.4 and 1 g E') in the chemostat fermentation running at a dilution rate close to 0.15 h'. In their study, they found that quick response of substrate uptake or consumption rate was independent of glucose intensity, whereas, process related promoter genes showed a dose related behavior. Successive glucose pulses can be done using an onloff-feeding strategy. Stress situation is increased in the oscillation process and fast perturbation frequency makes the stress even worse However, the disadvantage of perturbing the culture is that no subsequent pulse can be applied until the new steady state is reached. Single glucose pulse, on the other hand, were normally carried out by sudden injection of a glucose solution into a glucose-limited chemostat and results in sudden relief of carbon- limited conditions and thus in a sharp increase of the oxygen uptake rate. To quantify metabolic responses to nutrient perturbations, a fast sampling procedure is desired where metabolites have to to be tightly followed with each perturbation. First significant changes in gene expression were reported only visible between 120 and 210 s in S. cci'cvi.ciae 19], whereas for E. coli the nucleotides do not change significantly during the perturbation

experiments. Taken all the other perturbations, like two different doses of single glucose pulse 13], or a combination of oxygen and glucose all these can provide deep insight into how different environmental factors influence cell population heterogeneity and whether there exists such a thing as an optimal level of heterogeneity.

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Chapter 2

Bioprocess consideration—A way to simulate heterogeneity in controlled fermenters

Heterogeneity results from poor mixing inside the reactor both spatially and temporally often leading to conditions in which substrate concentrations andor physical parameters oscillate thereby continuously exposing the cells to gradients of for instance nutrients, oxygen and pH When it comes to industrial bioprocesses where a controlled cultivation of microorganism is central to maintain production efficiency and quality, understanding of the physiological property of living cells and maintaining optimal growth conditions is of crucial importance since population heterogeneity has a significant impact on the product consistency and quality. Ideally. cell heterogeneity would not be expected if the growth condition were well controlled. However, cell-to-cell differences, including differences in cell cycle and cell aging. contribute to the generation of phenotypic heterogeneity that exist at all the time, even if the microenvironment is constant, and only become relevant or important during environmental perturbations Heterogeneity, to some extent, is a beneficial solution during environmental perturbations either with higher growth rate or strength competence to boost population level fitness and process robustness In the present chapter cultivation techniques will be overviewed and special consideration will be given to process related cell physiology.

Cultivation methods and process related heterogeneity

Fermentations in different reactor configurations can be designed and considered as a representative heterogeneous microenvironment. Cell population inside the well- mixed reactor is rather homogeneous. but more heterogeneity could be found in reactors with less mixing efficiency. Reasons can be that parts of the bioreactor may provide optimal growth conditions whereas cell growth condition becomes harsh in other parts. Also the inoculation and operation with regard to. for example. inoculation time and substrate concentration may have major influence on cellular metabolic and physiological states Conditions like high substrate concentrations in high—density fermentation ma produce various undesired subpopulations with byproducts. This kind of side effect is magnified through reactor enlargement where subpopulation is found The heterogeneous environment in large-scale fermenters may lead to the repeated cycles of production.. re-assimilation of overflow metabolites and repeated induction/relaxation f stress responses resulting in reduced

biomass yield and productivity The three major and popular cultivations are batch, fed- batch and continuous, each of which has a distinct characteristic.

Bioprocess consideration

Batch cultivation

Batch culture has predominantly been used for years in the biotech industry in tenns of operability, reliability and economic considerations. Batch cultivation in a shake flask is the main cultivation used at bench level experiments. It is a closed simplest process where nutrients are added at once into the initial medium. It is not ideal for characterizing cellular processes, since when growth in a batch mode the physiological state of the cells is constantly changing because of adaptation to the changing environment{53, 16], indicating a changing gene expression and a varied physiological response to environmental perturbation

Fed batch cultivation

Fed-batch is a high-density batch process, which is based.on feeding with a growth limiting substrate to a culture. Now this cultivation becomes one of the most effective and preferred techniques in industry to maximize productivity. From aspects of feeding strategy. feed components and feeding outcome, means have been developed to avoid energy overflow (side products) and oxygen limitation (which could make up to 10% of the total working volume Feeding can be achieved in a certain way. including feeding exponentially at a constant rate. stepwise increase and feedback control (based on pH or dissolved oxygen measurements (pH-stat or DO-stat). Surface additions, although not optimal, is still a common way used in industry. Feed doesn't have to be only glucose. other components like amino acid 22] and ammoniurn can also be fed into the culture when needed. Feeding profile iviav influence growth rate. production rate, outer membrane permeability and protein compositions and periplasmic protein leakage Leakage. distinguished fi-om cell lysis. is defined as the passive/selective passage of proteins throu the outer membrane 124]. Protein leakage seems to be a process related phenomenon. which is found to be twice as high as in chemostat cultivation compared to the constant leakage level in fed-batch [36] and a higher feed rate correlates with a higher protein leakage 106]. To minimize the influence of leakage. membrane proteills as OmpF and LamB are identified as the main contributors I U6]. This optimitation i preferable in industrial production since the periplasmic space provides a more oxidative environment than cytoplasm that facilitates disulfide bond formation and also a decreased proteolytic activity. Improvement of periplasmic production has been considered from optimizing the induction parameters in E. coli host strain

A stress condition usually comes with glucose starvation and the present of cell death (=16%)(and cell lysis in the late feeding phase are mostly due to the high density of the culture over a long time scale. The increase in mixing time during scale-up inevitably leads to a formation of gradients in the concentration of the limiting substrate The consumption of the residual glucose normally leads to elevated levels of ppGpp and defensive proteins in E. coli within one minute and specifically, significant changes in gene expression were reported between 120 and 210 s in S. cerevisiae Based on these findings, selection and study on specific stress relevant genes open up the way to look into cell physiology, which could help to understand and circumvent some problems in the bioprocess Reactor performance is influenced on a large scale by mixing inefficiency inside the reactor both spatially and temporallv Scale up le oscillating conditions includes gradients of substrates. oxygen and pH, that cells are continuously exposed to in large reactors. Such an

inhomogeneity is amplified in fed-hatch cultivation in terms of substrate gradient. More than 10,000 fold concentration difference between the feed solution and the bioreactor broth in large-scale reactor generates varied gradients depending on the location of the feed 4]. Glucose gradients generated by improper mixing in a E. coli fed-batch fermentation at top. middle and bottom levels in a 22 m fermenter affect heterologous protein production. product yields and cell viability. induce stress responses of four stress sensitive genes and bacterial cells differentiate even morphologically near the feeding point [3].

Scale down devices

The scale up of bioprocesses from bench scale to large scale leads to lower yields. lower production and an ele\ ated by-product accumulation The aim of designing a scale down method is fairly clear that potential conditions encountered in scale up level can be foreseen and considered at the lab scale. \iaii\ kinds of scale down simulators were thus invented in the lab to generate certain degree of inhomogeneous condition with respect to magnitude of the substrate gradient. the period of oscillation and the circulation time 1291. Cells are either circulated between t\vo connected well—mixed stirred tank reactors 1 STR—STR) 130]. or a round circulation from a STR through a plug flow reactor (STR 1. 129]. The practical and the feasibility of this plug reactor is validated with different residence time setup and compared to a well mixed 5 liter reactor and 20—m With some biornass loss, in general, the plugin reactor is a good candidate of studying large-scale heterogeneity at bench level. Of many-presented scale down studies, one or two compartments models have

Bioprocess consideration been widely utilized (Figure 2.1). in this PhD study the plug flow reactor (PFR) is transformed into a 12-meter recycle loop (diameter 0.005 m) and used to simulate perturbation by adding the feed (glucose) to the entry point, with the residence time of 79.8 s aiming to prevent anaerobic fermentation at a short resident time Schweder et at. (1999) found in a similar configuration with a residence time of 10 mm that only 13 sec of exposure to low oxygen at the presence of high glucose is sufficient to cause mRNA level accumulations of overflow byproducts Compared to nonTlal fed-batch fermentation, concentration gradients of glucose and oxygen are generated at the inlet of the recycle loop. The glucose variation at different locations of the SDR, as representatively diagramed in our previous study [is in the range between 0.01 and 0.25 g/L. Statistic interpretation can be found in and comparison was done between a 2-m reactor and the scale down reactor. Foaming fonnation is suiprisingly reduced in this kind of set up with both residence time of I OOs and 200s{ 134]. which bring its potential application in foaming control to improve reactor performance. Differed ith a recycle loop, the pulse can be performed t'wougk the P from the o of the veactor whk1 seems have broader application.

Scale-down effect at the level of cell growth is reflected as a decrease of the biomass yield and by-product accumulation compared to a well-mixed reactor However, positive effect has also been observed in the way that microbial cells exposed to inhomogeneous conditions tend to exhibit Io\ cr membrane permeability and a higher viabilitv which is the fact occurring in the large fernienters. Heterogeneities induced by scale-down may also affect protein balance of synthesis and degradation An increase of protein degradation upon either glucose or oxygen starvation is controlled by the stress signal guanosine tetraphosphate (ppGpp) formation For those damaged proteins. proteolysis would remove them and rene\v the cell body with favored built— up material.
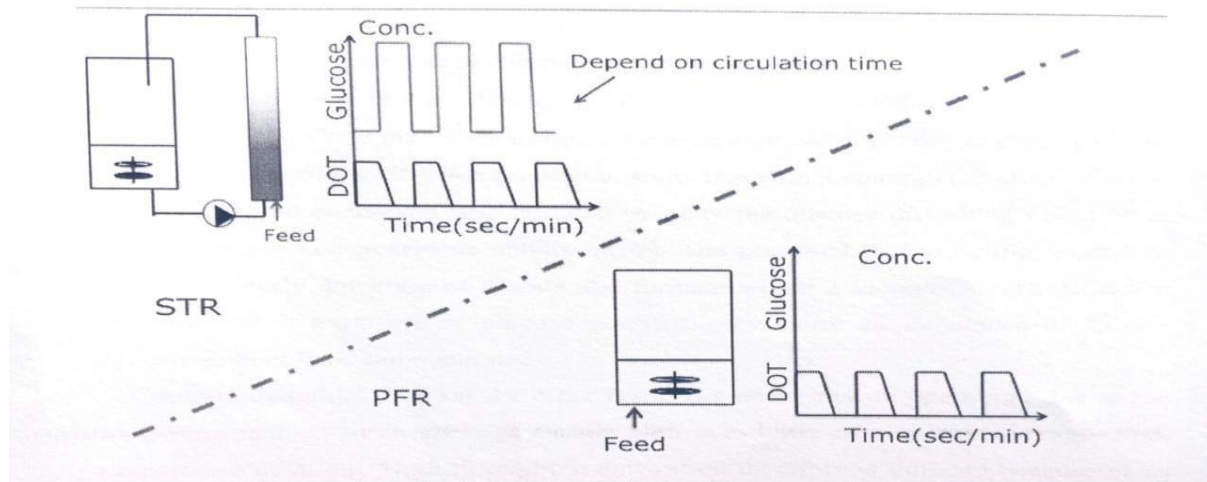
Figure 2.1 Principle of the scale-down reactor indicating the concentration fluctuations with respect to glucose and DOT experienced by an individual cell circulating through STR (upper left panel) and PFR (lower right panel). For STR. periodic cycle of excess and lack in the supply of glucose and oxyttell is illustrated the !ucose fluctuation, which is depending on the circulation time, has been attributed to cause DO depletion. The plots show schematically the concentration fluctuation with respect to glucose and DO concentrations experienced by individual cells in both kinds of reactors.

Chernostate

The growth environment in batch cultures is continuously changing. Those growth rate- dependent factors tend to obscure the identification of genes that are specific to a particular experimental treatment, mutation, or physiological condition. One way to address these issue' is to use chemostat cultures where the growth condition is rather stable. Chemostat fermentation is a good tool to study the physiology of cells since it creates steady environment where cell growth rate is set equal to dilution rate atler a certain time range, and then intrinsic variables in this open system are remaining constant.

Genes specific to the particular limitation are grouped into an induction or repression pattern 16. 137]..\ uniform dividing and non—dividing manner in the exponential and 4 growth phase has been cxperimentall\ observed in batch culture and dividine and non—dividing subpopulations of different GFP expression have emerged after transition of the stationary phase cells into a fresh LB medium 138]. Ste state growth—limiting cells are not able to up—regulate their gro\vth rate immediately upon transition to glucose non—limiting condition in batch Transcriptional follow up studies subjected to two different doses of single glucose pulse 13], or to a combination pulse of oxygen and glucose reported that there were different response patterns upon perturbation in the Saccharonnces cerevisiae chemostat culture due to sensitivity to the available carbon. Further, the choice of dilution rate may alter some of the gene expression pattern in either glucose or ammonia shortage An oscillation study based on a combined method of scale down setup and chemostat was initiated to study the glucose disturbing effect on E coli in aerobic and anaerobic condition The proposed fast sampling procedure enables an early detection of acetate and forniate within 2 seconds, which dedicates that even short exposure to glucose gradient, may cause an imbalance of E. coli glucose metabolism and respiration.

Continuous cultivation, on the other hand, is complicated in operation, due to the long time needed to reach steady state, high possibility of contamination, medium preparation cost. etc.

High throughput cultivation therefore is initiated because of its massive information and low costs A micro-scale system named as microfluidic device recently turns up to ease a chemostat process with less medium consumption and precise control. Continuous-flow microfluidics have successfully been used in condition control in characterizing physiological behavior 141]. in persistence study and in tracking of single cell lineages on protein level Without sampling, online detection of fluorescent proteins in parallel small-scale continuous cultivations has been developed in microtiter plates 145]. real time data of :2m\vth kinetics and GFP production for different strains ha\•e been gathered without iliterruption of growth. Microarray, for instance, was preferable used in this kind of high throughput genome-wide experiments, since a large number of genes can be statistical analyzed simultaneoush'.

Chemostat is a poor but not starved process 146, 16] where cells are less stressed than in batch culture and cell viability remains quite high 147]. The interlink between chemostat and batch mode is that chemostat repi-esents a way to maintain the cells at a particular point in the batch process Ho\\e\ er. cell status is not the same in elL!lcr hatch or its comparable chemostat under nutrient limitation. Evidence showed that stress responses that are not activated in chemostat are activated at the point comparable in the batch The high level of the residual nutrient allows the cells to maintain in unhealthy but not stressed condition.

Further. chemostat offers a great advantage of reproducibility and accuracy over the other techniques. One thing that needs to be kept in mind is that not a single method is pure perfect. both the pros and cons of the current prevalence miniature bioreactors are detailed in a review The availability of those cultivation techniques offers an excellent window to study the population behavior that occurs in nature. On the other side, it is convenient to use the microbial population as physiological tracer for estimation of the bioreactor mixing and transfer efficiency. This idea is also reflected throughout the thesis.

Influence of byproduct accumulation

Most often, conditions like glucose or oxygen gradients, overflow or mixed-acid fermentation, would cause production of some highly undesirable byproducts, because of the redirection of biomass formation to useless energy consuming pathways and the inhibition on growth. Mixed acid fermentation usually occurs under anaerobic conditions and the accumulation of formic acid, acetate, lactate, ethanol, and succinate is detectable 2s after expose to glucose and 02 gradients In aerobic conditions the overflow metabolism initiates when pyruvate are not oxidized through citric acid cycle and respiration. All these byproducts can easily be re-consumed during the process There is no doubt that cycles of synthesizing and consuming of these mixed acids when exposed to oxygen sufficient:oxvgen deficient zones result in a lower biornass \ields, especially at large scale 85].

Using acetate as an example, the production and inhibition effect of acetate is specific to strains, medium composition, cultivation conditions or feeding strategy. growth rate and pH In E. co the "acetate switch" is physiologically defined as the moment when acetate dissimilation excretion/production equals its assimilation/utilization; experimentally it is where extracellular acetate peaks during aerobic growth in minimal medium with glucose used as the sole carbon source; transcriptional!v. initiation of acs transcription is a primary player among many other pai- ticipants The gene acs, encoding acetyl coenzyme A (acetyl-(oA)

svnthetase. is responsible for converting excreted acetate to acetvl-CoA. Interestingly. the activation u's i mediated through quorum ,cn according to the cell nutritional condition, rather than the extracellular acetate concentration 154]. The rate at which acetate forms is directly related to the growth rate and the glucose consumpt rate. E. coli generates acetate when glucose is the limiting substrate and cells grow above a threshold growth rate. Apart from the diversion of carbon that might be used in the synthesis of biomass or the protein product, acetate production causes a number of other problems since it inhibits growth even at very low concentrations, e.g. 0.5 g/l inhibits protein formation, affects proteins and genes involved in stress response and relation process The growth inhibition for E. coli expands more significant in a defined medium than in a complex medium in a shake flask experiment and more severe in recombinant strains than in wild type strains Therefor. growth on glycerol, which is known not to provoke acetate accumulation is of interesting as alternative to carbon to be explored in detail. And again, not all the strains, exemplified by E. coli strain MC1O6O and JRGJ06J re capabie of shithng to grow on acetate.

E. coli can utilize acetate via the glyoxylate pathway, thus the total acetate concentration in the medium is the result of both production and consumption. In the context of the mixture of glucose and acetate glucose is preferably used in the first place over acetate in a batch fermentation, whereas, simultaneous uptake of both substrates is reported in chemostat at a dilution rate below 0.3 h At a dilution higher than that, E. coli produce more acetate from acetyl- CoA generating huge amount of ATP and NADH2 to meet a higher energy demand in order to keep a functioning cell factory. This suggests that the acetate inhibition effect can be minimized by its consumption, besides the way of preventing overflow on a lower glucose concentration. Trials on genetic deletion of the acetate pathway minimized acetate accumulation, but elevated other byproducts were found Some other process modification approaches of reducing acetate in

E. coil fermentation are still in progress to meet a variety of production demand. The ad\ ancement in recent developed approaches has been nicely reviewed by Mark et al

By considering the metabolic routes employed by E. coli for the metabolism of acetate (Fig. 3.1). Harry Hoims and Peter Bennett were the first to provide the evidence that E. coli can not use acetate as sole source and energy until it synthesize the enzyme of the glyoxvlate bypass, which is repressed during the growth on lucose 138]. Surprisingly, the addition of chioramphenicol at the end ol the growth inhibits synthesis of ICL (isocitrate lyase) enzyme. the existence of which is reported to he necessary when growing on acetate. The conforrnational changes and domain shifts of the ICDH (isocitrate dehydrogenase) in conserved TCA cycle is central to sticcessful adaption and growth on acetate [161].
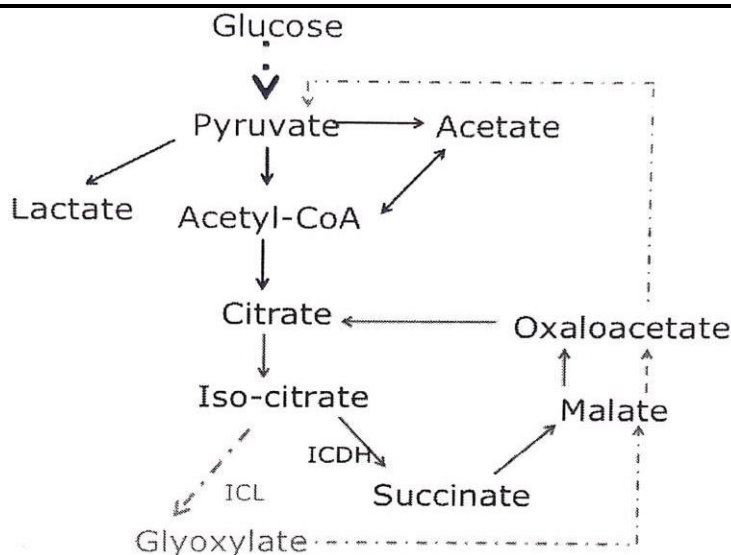
Figure 2.2 Metabolic network of E. coli growing on glucose or acetate as the sole carbon and energy source. Abbreviations: ICDII, isocitrate dehydrogenase; ICL, isocitrate lyase. During growth on acetate. ICL meditated glvoxvlate bypass and ICDH meditated TC'A cycle are in direct competition for isocitrate.

A similar toxic metabolite in cultures as acetate is formic acid with high membrane permeability, which is more toxic than acetate and inhibits DNA synthesis and cell di\.ision 1621. Both acetate and D-lactate are re-assimilated much more quickly than formate hen E. coli cells re-enter an oxygen sufficient zone, leading to an accumulation of formate in the culture mediurn Therefore formic acid used to be considered as an indicator of anaerobic zones because of its slow rate of re-uptake. It has been proved by the oxygen limitation study in the STR—PFR setup that formic acid will not be accumulated once trace element solution of selenium, nickel, and molybdenum is added to E. coli growth medium However, instead of formic acid an elevated amount of lactate is recorded in a shake flask test.

Process monitoring

The tight control over the physical and chemical surrounding environment of the cultured microorganism ensures a consistent and optimal pi-oductivitv. therefore, a controlled cultivation becomes a central core to hioprocesses whcrc both online and offline monitors are highly needed. A range of parameters most o controlled is pH. dissolved oxygen tension (DOT), optical density. Llgitation. foaming, exhaust gas composition and substrate concentration. etc Specific fermentation strategies aiming at minimizing acetate nd elevating production. like glucose- feeding approaches, controlled growth rate and alternative feeds of gl\ cerol, have been demonstrated with cei'tain degree of success. Although undesirable low production and non- optimal growth always weaken the strength of these methods, focus on process monitoring still have not shifted totally. In order to get reliable online measurements detecting sensors or probes with high integrity and stability cannot be omitted. These sensors in general should be able to endure sterilization, be durable for long-term usage and easy for calibration. Polarization with electrolyte solution and calibration of the probe is needed, further, exchange of electrolyte for each new run. The DO probes membrane must also be inspected prior to use. Similar rules apply for the pH probe. Ensure pH probe has enough electrolytes, in good condition and carefully calibrated is crucial for a successful experiment.

Indirect measurement on a representative sample of the process means a lot to get a proper estimation and evaluation of process supervision. ideally, as many online measurement as possible is preferred, since the real time information is provided.

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Chapter 3

Short outline of Experimental design

Scope

1. Demonstrating   the applicability of the reporter   system in addressing the physiological state of the cells in respect to substrate limitation in batch, fed batch, and chemostat.

2. Presenting a destabilized variant of GFP as reporter in addressing cellular status together with the application of fluorescence stains;

3. Transient behavior in front of nutritional disturbance. Stimulus-response experiments are performed to monitor cellular physiological responses.

4. An insight into heterogeneity in robust fermentation processes.

Experimental Outline

Batch fermentation

Batch processes have been intensively investigated for a long time but there are still some variations among different batches. Among others, the physiological state of the cells when grown in a closed batch-culture is constantly changing because of adaptation to the changing environment, indicating a changing gene expressions and a varied physiological response tb environinental perturbation. Rapid staining procedures provide quick information on cell states and therefore enable fast initiation of process control. It is important to analyze heterogeneity by distinguishing the stages of functionality of cells probed by the staining procedure such as cells with certain degrees of membrane integrity, distinct enzyme activity, or different energy status. In Manuscript 4, the combination of ' SYBR Green, P1, DiBAC and RSG as indicator of respiration activity and n. potential are used to assess cellular viability of bacterial cells. The application of thcse staining dyes in a E. co/i population gives us better insight into diverse physiological states of bacteria cells in a batch cultivation.

Scale down fermentation

Bioprocess scale-down simulators are used to investigate the phenomenon occurring in trial scale cultivations. The aim is to simulate the large-scale conditions, characterized by long mixing times introducing local gradients into the bioreactor, in laboratory scale bioreactor. Typical scale down (SD)

configurations has been described in chapter 2. It is a nce tool to investigate the bacterial response to spatially structured environment. Mauscript 1 deals with problems associated with the concentration gradients that exist in i bioreactors. The situation was mimicked with a two-compartment bioreactor svs m consisting of a stirred tank reactor (STR) connected to a 12-meter recycle loop to the poor mixing scenarios. Oxygen was provided in the well-mixed stirred tank and Short outline glucose is fed in the beginning of the recycle ioop, thus a zone featured with glucose rich and oxygen limit in the loop is generated. The response of E. co to transient exposures to glucose gradients under controlled aerobic and anaerobic conditions was investigated and analyzed in this long-term transit exposure to nutrient limitation.

Pulse oscillation

Cell physiology studies over a range of perturbations are necessary to make accurate predictions of how external variants cause cellular imbalance. The perturbing agent is generally chosen to be glucose, because glucose is the most preferred carbon source for many organisms and, accordingly, changes in glucose availability often have profound consequences in many types of cell gene expressions.

(1)   Single pulse

Some other ways that have been used to study physiology responses to substrate gradients are those in the chemostat fermentation context but perturbation with substrates pulses, followed by a fast catching up sampling. The metabolic and physiological responses to the perturbations, together with gene expression analysis are focus in this short time oscillation. This is accomplished by injecting a glucose pulse in one shot at two dilution rates of 0. 1 h and 0.5 h vespectively, in Manuscript 3.

(2)   Programmed pulses with fixed frequency

Considering the period of pulse effect in the given steady experiment, a rather longer observation window is opened for another experiment, which is programmed for long time substrate oscillation using the dedicated matlab code. The dilution rate in this study is 0.14 h' aiming to create a severe starvation environment and minimizing side effect of byproducts such as acetate. Gene expression at the phase transition from batch to the switch of continuous cultivation and at the stabilized culture is evaluated, as well as membrane permeability (Manuscript 1 and Manuscript 2).

CHAPTER 4

STRAIN CONSTRUCTION

Attempts were made to construct E. co/i biosensor strains carrying chromosomal inserts of GFP under the control of a ribosomal promoter and stress responsive promoters, respectively. However, constructed chromosornal reporter was proved unsuitable for this project as the GFP signal was too low to be detected by

the flow cytometer, hence, it was decided to continue the work with plasmid based biosensor, that is MG 1 655/pGS2OPrrnBGFPAAV and MG 1 655/pGS2OPfisGFPAAV.

Strain construction

Previously, cell growth control mechanism has been taken into consideration concerning heterogeneity, cell behavior and bioprocess approach, this chapter will describe strain construction and its signal verification.

Biosensor construction

Unstable GFP amplification

The gJj(AAV) gene, encoding the unstable GFP variant GFPAAV with reported short half-life around 60 mm in Escherichia co/i [was PCR amplified from plasmid pBSKPsosGFPmut3 (pUC origin, ampicillin resistant marker, 50 sg/ml) (Epoch Biolabs Inc., US) by using primers GFP-up and GFP(AAV).

Construction of plasmid pGS2OPsosGFPAAV and plasmid pGS2OrpoSGFPAAV

Plasmid backbone pBSKPsosGFPmut3 carrying the E. co/i promoter sos was digested with NdeI and sgrDl and subsequently ligated with the same sites of gt resulting in plasmid pBSKPsosGFP(AAV).

Later, gene cassette Ps0sGFP(AAV) flanked by restriction sites of Noti and .sgrDl derived from this plasmid was included into a pre-digested pGS2OPrrnB plasmid (colEl replication origin, — 10 to 15 copies/cell, chioramphenicol resistant marker, 25 ug/ml) (Epoch Biolabs inc., US), creating pGS2OPsosGFPAAV. This plasmid furthermore contains a strong rrnB T transcription terminator to p1-event readthrough from the promoter.

Construction of plasmid pGS2OPrrnBGFPAAV, pGS2OPrpoSGFPAAV and pGS2OFi sGFPAAV

The 760 bp PCR product of gf, was digested with *a'cI and sgrDl and subsequently inserted into the same sites of the low copy number plasmid backbone pGS2OPrrnB, which is carrying the Ecu/i ribosomal promoter rrnB PIP2. Thus. the resulting plasmid, pGS2OPrrnBGFPAAV. expresses GFPAAV under control of the rrnB promoter.

pGS2OPrrnBAAV was digested with Notl and Ndel to replace the craB promoter region with the

E. co/i Jis promoter. Thetis promoter was amplified from the Li co/i chromosome by using the primers FisP-SD (the primer contains an optimized SD sequence) and FisP-up. The 300 bp PCR product was digested with Null and Ndel and ligated into the vector fragment. The resulting plasmid was called pGS2OFisGFPAAV.

RpoS promoter, similarly, was first amplified by PCR amplification using primer RpoS- FW and RpoS-RW, followed by restriction using NotI and Ndel. Then, ligation of promoter rpoS into vector fragment containing the same restriction sites resulted the plasmid pGS2OrpoSGFPAAV.

Construction of plasmid pGS2OPrrnBGFPmut3

A NotI-NdeI fragment containing the rrnB promoter from plasmid pGS2OPrrnBGFPAAV was cloned into the same sites of plasmid pGS2OPA 1 O4O3GFPmut3 thereby replacing the PA 0403 promoter.

Plasmid transformation and verification

Table 4.1 Primers used for plasmids construction in this study

Primer	Sequence (Relevant restriction sites are underlined)
GFP-up	5-ATATACATATGCGCAAAGOTGAAGAACTGTTTAC NdeI
GFP (AAV)	5'-CTCTCCGTCGCGATTAAACTGCTGCAGCGTAGTTTTCGTCGTTTGC Sgr DI TGCAG GC C TTTTG T AC A G TTC A TCC A TG CC C
FisP-SD	5'-CCCCCATATGTAAATCCTCCTTATTGAAATGACCATACTGTGACT NdeI GCAAGGC
FisP-up	5'-CCCCCGCGGCCGCCTCACAAAAGGCACGTAAATTTGCCG NotI
RpoS-RW	5'GCACGTACGCGGCCGCTTCGGGTGAACAGAGTGCTAAC NotI
	5'-CAGTCCACCATATGGTGGCTCCTACCCGTGATCCCTTG NdeI

Strain construction

The generated plasmids were transformed into Genehogs and finally to E. co/i wildtype strain MG 1655 by electroporation using a Bio-Rad Micropulser according to the manufacturer's instructions and stored at -80°C. All the promoters and GFP region were fitted into the same restriction frame (NotI-promoter-NdeI-GFPAAV-sgrDl-T 1- Sac]) for easier use in future. Plasmid verification was done by enzyme restriction and sequencing. A series of primers used for construction is listed in Table 4.1.

Construction of chromosomal reporter MC 1000: :PrrnBGFPAAV

For the plasmids constructed above, pGS2OPrrnBGFPAAV, pGS2OrpoSGFPAAV and pGS2OPsosGFPAAV, the promoter and the protein gene region of each plasmid was extracted by restriction using Not! and SacL followed by the ligation into the template plamid pUC18R6KT- mini-Tn7T (originally carrying ampR, and the gene of KnR was extra inserted between the Tn7R and Tn7L region). This operation generated plasmi ds: pTn7PrrnBGFPAAV, pTn7rpoSGFPAAV and pTn7Ps0sGFPAAV. Clones had been verified by restriction and sequencing. After confirmation of the right clones, four-parental filter mating was made to insert the target gene of promoter and fluorescence protein into the chromosome of E. co/i MC 1000. Relevant antibiotic resistant property is shown in Table 4.2.

Table 4.2 Strains and plasmids used in mating

Role	E. coli Srain	Plasmid	A	K	G	S	C
Recipient	MC100		-	-	-	+	-
Donor	Mt 102::pir	pTn7PrrnBGFPAAV	+	+	+	+	-
Helper	HB101	pRK600 (mob)	-	-	-	-	+
Helper	DH5a	pTNS2 (TnansABCD carrying)	-	-	-	-	-

Rsulted   New MC 1000   -   +   -   + -

Note: A=Arnpicillin: K=Kanamvcin: G=Gentaniicin: S=Streptomycin; C=Chlorarnphenicol. pT\S2 was derived from referenee

Later. recombinant strain with the right resistant property had been selected, followed by a serial dilution procedure of five days at 42°C without antibiotics in LB medium aiming to lose the parent plasmid after several generations. Then, colonies appearing on the selective plates were tested by PCR amplification. Results showed that there was no insertion between the phoS and g/nS sites on the chromosome (see simplified principle in figure 4.1), indicating that random insertion had occurred during the process. One of the reasons might be that the transposon sequence was unfortunately incorrect.

To make a new functioning Tn7 transposon, a 1969bp gene fragment containing the temperature-sensitive replication and the ampicilin gene was obtained from ampicillin resistant plasmid pKD46 by using primers pKD46 oriRlOl XbaI FW and pKD46 oriRlOl NcoI RW. Another 2736bp Tn7 fragmenet was PCR amplified from template pUC18R6KT-mini-Tn7T (ampR) with primers PUC18T Tn7T XbaI FW and PUC18T Tn7T NcoI RW. Thus, the ligation of the two resulting fragments created a new Tn7 transposon of 466 lbp (amp' Next, gene cassette of NotI-rrnBGFPAAV Ti-Sac] was moved from pTn7PrrnBGFPAAV into the same sites on this new Tn7 transposon (temperature sensitive replicon, oriRiOl, 30°C, ampicilin resistant, 50 .tg ml). getting a new pTn7PrrnBGFPAAV. A kanamycin resistant gene from pUC18R6KT-rnini-Tn7T was later inserted between the C and BaniHi sites on the new pTn7PrrnBGFPAAV. Again it had been verified by sequencing. Parallely, pTn7PrpoSGFPAAV, pTn7Ps0sGFPAAV were all construted. But, from this point on, only promoter rrnB has been chosen to proceed with by considering the promoter strength (see next section of signal verification). Primers used for amplification are listed in Table 4.3.

Table 4.3 Primers used in creating the new Tn7 transposon

Primer	Sequence (restriction sites have been underlined)
pKD46oriR101 Xbal FW	CGACTCCGTCTAGAGTCAGACCAAGTTTACTCATATATAC
pKD46orilR101 Ncol Rw	CGAAGAATCCATGGGTATGGACAG
PUC18TTn7T Xbal FW	GTTCACGGTCTAGACGTTACCAATGCTTAATCAGTGAGG
PUCl8t Tn7T Ncol RW	CGTACAAGCCATGGGCTCACATGTTTCTTTCCTGGGTTATC

Then the new Tn7 plasmid was electroporated into E. co/i DH I OB, after 6 hours regrowth at 30°C in SOC medium, plating was carried out and selective plates with antibiotics of ampicillin and kanamycin were kept at 42°C overnight to block plasmid replication. After this was done, three sets of primers (see Table 4.4, Figure 4.1) were used for PCR verification of the right clones (two colonies were tested, shown on Figure 4.2 left panel). After PCR verification of the right integration into DH1OB, chromosomal integration of genes of interest into E. coil MC1000 was facilitated by phage P1 transduction. Sixteen colonies had been tes\ed using primer set of phoS Fw and gimS Rw and the result is shown in Figure 4.2 right panel). Thus chromosomal reporter MC1000::PrrnBGFPAAV was made.

Table 4.4 PCR verification of the chromosomal integration

Primer	Sequence	PCR Product Size
phoS FW	GGAACTGGAACAGACCTTCCTGAG	
glmS Rw	CGATCAGTACCCAATCGCGCTGGAAG	
pTn7L	ATTAGCTTACGACGCTACACCC	
phoS Fw-Tn7L		
glmS Rw-Tn7R		
phoS Fw-glmS Rw		

Note: -. denotes no insertion between sites phoS and gimS. otherwise, +. The cooresponding size using each primer set is writing as PCR product size.
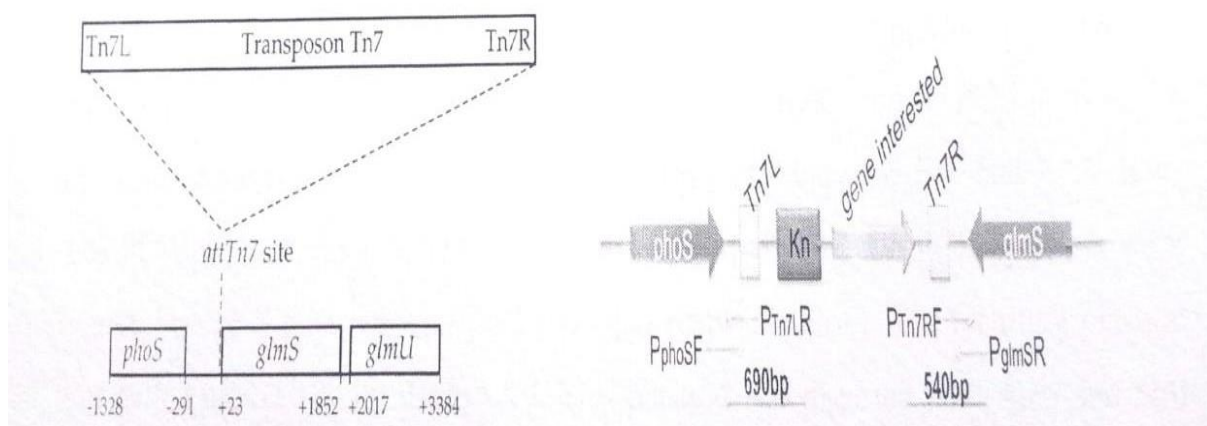

Figure 4.1 Physical maps of gene phoS and ghnS on E. coli chromosome (left panel), primer targets and physical map of the inserted genes (right panel)
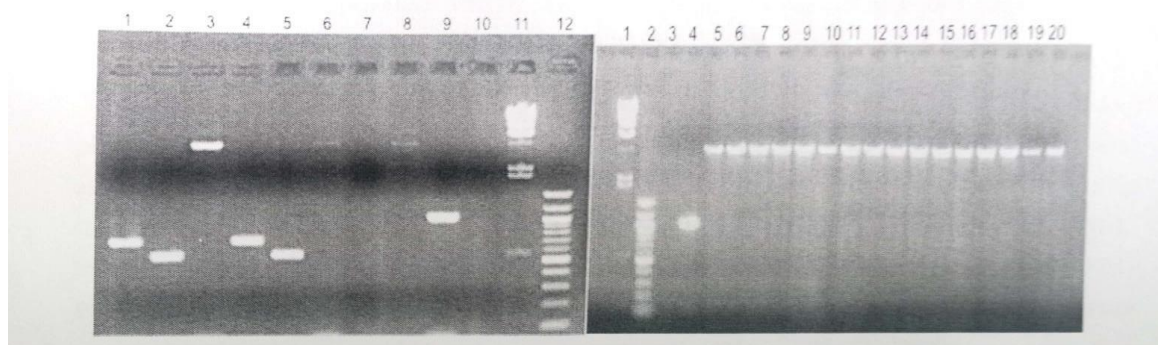

Figure 4.2 PCR amplification result from chromosomal integration. Lamda marker and 1kb marker were used. Left panel (test for DHIOB): two colonies (lane ito 3 and lane 4 to 6) were picked and tested with three sets of primer (listed in table 4.4). Lane 1, colony I tested with primer pair phoS Fw-Tn7L, lane2, glmS Rw-Tn7R, and lane 3, phoS Fw-glmS Rw. Same physical order was displayed for colony 2. Unfortunately one PCR product was missed on lane 6. From lane 7 to 9, wildtype strain using same primer order as the control was loaded. No bands appeared on lane 7 and 8, showing no insertion was made. A band about 1kb appeared on lane 9, confirming that no insertion was created between physical sites ofglmS and phoS. Lane 10 is the negative control for PCR amplification. Lane 11 and 12 loaded with marker. Right panel (for MC

1000): colonies were only tested with primer set of phoS and gimS. From left to right, markers (lane I and 2). negative control (lane 3). wildtype control (lane 4) aHd 16 clones from lane 5 to 20.

Construction of plasmid reporter pX I. OPrmBGFPAAV pX 1 .OPrrnBGFPmut3

pXl.OPrmBGFPAAV and pXl.OPrmBGFPmut3 were further constructed based on a 30.2 kilobase (kb) synthesized plasmid X .0 with approximately 3—9 copies per chromosome and stable inheritance [The cloning procedure for pXl.OPrmBGFPAAV is: The TOT1 terminator was PCR amplified from pUCI8T-mini-Tn7 by using primers TOT l-FW arid TOT1-BW. The PCR product was digested with Noti and BamHl. PrmB-gfpAAV 'vas PCR amplified from pGS2O derivatives (mentioned above) carrying this construct by using primers rrnB-FW and AAV-BW. The PCR product was digested with Ba and Apal. The Iwo PCR products were gated into pKS digested with \otl and Apal (triple ligation). The resulting plasmid was called pKS-

TOTI-PrrnBgfpAAV. This plasmid was digested with Apal and Sbfl and the TOT1- PrrnBgfpAAV fragment was purified and ligated into pXl.0 digested with the same enzymes (thereby removing the tra cassette of pXl.0). The ligation mixture was electroporated into E. co/i Genehogs. Subsequently the final plasmid construct was moved into E. co/i strain MC 1000.

Similarly, Gfpmut3 was PCR amplified from pUC18T-Tn7-PAlO4O3gfp by using primers gfpmut3-FW and gfpmut3-BW. The PCR product was digested with Ndel and Apal and ligated into pKS-TOTI-PrrnBgfpAAV digested with the same enzymes (thereby substituting the gfpAA V gene). The resulting plasmid was called pKS-TOT1 - PrrnBgfpmut3. This plasmid was digested with Apal and Sbfl and the TOT1- PrrnBgfpmut3 fragment was purified and ligated into pXl .0 digested with the same enzymes (thereby removing the tra cassette of pX 1.0). The ligation mixture was electroporated into E. co/i Genehogs. Subsequently the final plasmid construct was moved into E. co/i strain MC 1000.

Table 4.5 PCR verification of the chromosomal integration

Primer	Sequence (Relevant restriction sites are underlined or strikethrough)
TOTl Fw	CCCCC**GCGGCCGC**CCTGCAGGGCTTAATTAGCTGAGCTTGGACTCC NotI Sbfl
TOTl Bw	CCCCCGGATCCCGGAATAGGTACTTCAAGATCCCC BamHI
rrnB Fw	CCCCC**GGATCC**aggctgattggttgaatgttgcgc BamHI
AAV Bw	CCCC**CGGGCCC**TTAAACTGCTGCAGCGTAGTTTTCG Apal
gfpmut3 Fw	CCCCC**catatg**cgcaaaggtgaagaactg Ndel
gfpmut3 Bw	CCCCCGTCGA**CGGGCCC**TTATTTGTACAGTTCATCCATGCCG Apal

Signal verification of the constructs

The different types of reporters for F. co/i aimed to monitor different responses on population heterogen city were constructed, namely, plasmid pGS2OPsosGFPAAV.

pGS2OrpoSGFPAAV.   pGS2OPrrnBGFPAAV,   pGS2OFisGFPAAV.

pGS2OPrmBGFPmut3 pX 1 .OPrrnBGFPAAV and pX I .OPrrnBGFPmut3, plus chromosomal reporter MCI000::PrrnBGFPAAV. The signal verification was then carried out to confirm each construct is capable of delivering detectable signal. A FACSAriaTM III (Becton Dickinson, USA) flow cytometer was used to analyze the fluorescent signal and the results were analyzed by FlowJo software (TreeStar, Inc., USA). The flow system includes two lasers with minimum laser power of 10 mW with the specific wavelengths of 488 nm and 561 nm. The detection settings for the forward scatter and side scatter were 301 V and 316 V, respectively. The fluorescent detection channel of GFP (530/30 nm) was set at 520 V. Fresh samples of bacterial cultures were taken at indicated times, centrifuged, suspended in 0.9% NaCl solution and then analyzed by flow cytometry. At least 30,000 events per sample were counted to have a good view of population structure. Alternatively, CDF (Cumulative Distribution Function) plots, which displaying exactly the same information as histograms provide, were used to interpret the data. The fluorescence of a histogram distribution corresponding to any given percentile can easily be found on a CDF plot (see figure 4.3).
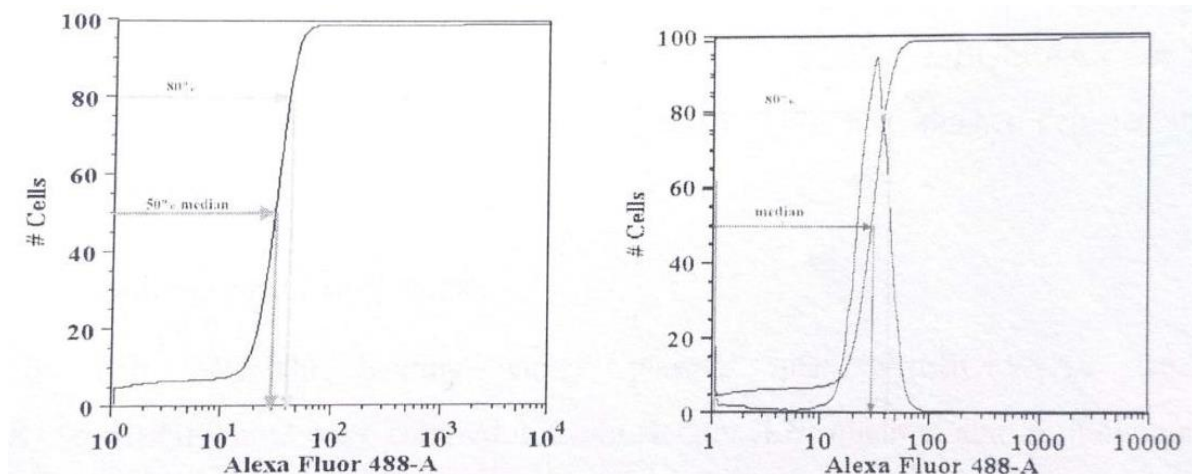

Figure 4.3 Demostration of a CDF plot (left) and the overlay of the CDF plot and the histogram. Same sample information is used.

1-liter medium was made by mixing 100 ml B\4 solution, 100 ml TM solution, and distilled water, supplemented with 5 g/rnl thiamine, 50 g!mI of iso leucine and valine. A detailed composition of the BM and TM solution is listed in Table 43. Agar 1 8 g/l was added to the medium when needed. Cells were grown aerobically at 37°C in minimum medium or in LB medium. Chioramphenicol 25 g/rnl was added to prevent contamination of fungal and other bacteria and bacteria that lost the plasmid will die out.

Table 4.6 Medium composition

Stock	Component (g/L)
Bm (10X)	K2HPO43H2O, 42.5; NaH2O, 10; NH4CI,20.
TM (10X)	MgSO47H2O, 2; FeSO47H2O,0.12, ZnSO47H2O,0.03; MnSo4H2O, 0.03; Nitrilotriacetic acid, 1.23.

For plasmids pGS2OPrpoSGFPAAV and pGS2OSOSGFPAAV, induction signal was quite weak so we decided to continue with pGS2OPrntBGFPAAV in later on construction experiments. Unfortunately, after insertion of naB promoter into F. coil chromosomal, no clear induction signal of chromosomal reporter can be found in the output data when using flow cytometly. Encountered by the failure signal reading from chrornosornal reporter, the big synthetic pX 1 .0 was considered. However, the GFP signal ofpXl.OPrrnBGFPAAV was also too weak to detect with flow cytornetry. One possible explanation is the very low copy of the reporter gene sitting on the chromosome or on the plasmid pXl.0. Plasmid pGS2OPrrnBGFPAAV and pXl.OPrrnBGFPmut3 were proved to have a better signal performance detected in flow cytometer.

Result of signal induction test

E. coli MC1000 bearing either plasmid pGS2OPrrnBGFPAAV and pXl .OPrmBGFPmut3 were cultured in Luria-Bertani (LB) medium and in minimum medium aiming to provide different growth rate in order to see the fluorescent shift in growth phases. Typical data extracted from the signal testing experiment were shown in Figure 4.4. The arrow indicates the order of the displayed samples start from early exponential phase till stationary phase. Data are plotted in regards of fluore intensity, forward scatter, side scatter and then comparisons were made in Cumulative Distribution Function (CDF) plots. Overlaid histograms offset are properlY used in order to distinguish the curves better.

In general an induced single GFP peak appeared in early exponential phase and then declined for the rest of the growth phase. showing that all the cells are propagating at an equal rate. Greater increment of fluorescence is seen in the case of pGS2O- based plasmid (Fig 4.4, A and B) than pXl .0-based plasmid (Fig 4.4. C and D). Possible reason medium is that the copy number of pGS2O per chromosome is larger than pXl.0, which is considered as 3—9 copies per chromosome Besides information obtained from fluorescent protein, the emitted light gathered from forward scatter (FSC) and side scatter (SSC) contains information on cellular property, such as cell size is reflected by FSC and cellular/particle density is by SSC. The principle is that microorganisms could scatter and bend light, thus light scatter can be used to provide information on metabolic and physiological states of cells. Compared with GFP expression, similar inducement is shown in FSC, which is an indicative of cell size changes. It infers that cell becomes bigger in early exponential phase and then after goes back to small size when growth is slowed down, since the size of E. coil depends on the growth phase and the nutrients available in the medium Cell size when grown in LB medium is limited by the scant amount of carbon and then is the mount of usable amino acid. so cell size starts to decrease even in exponential phase
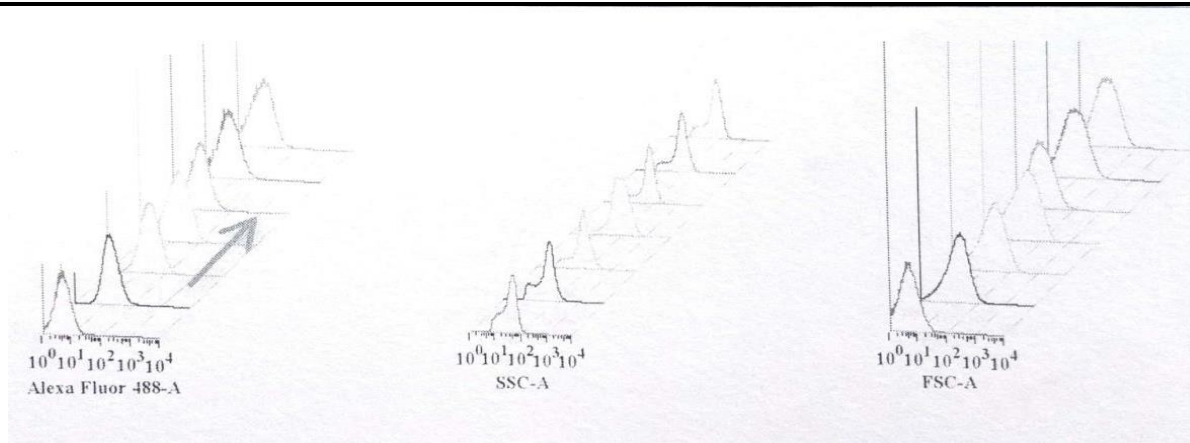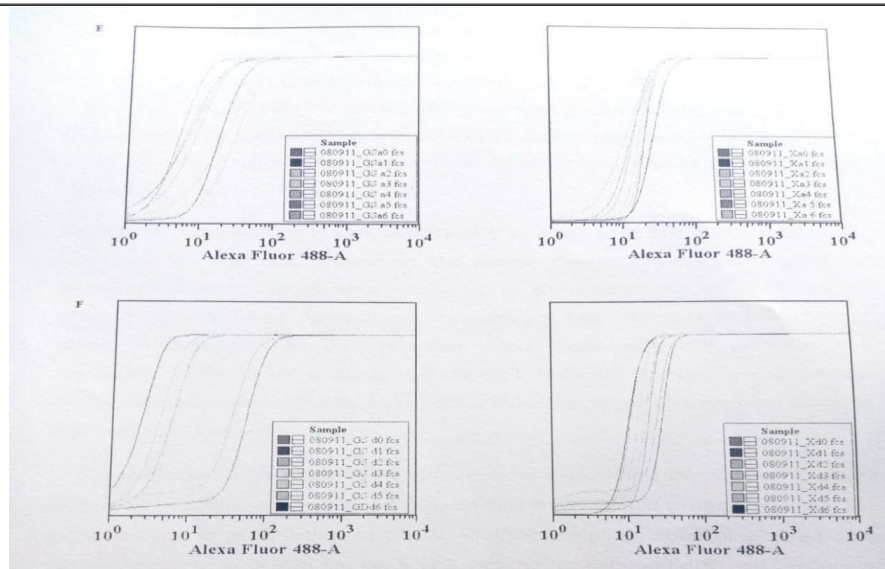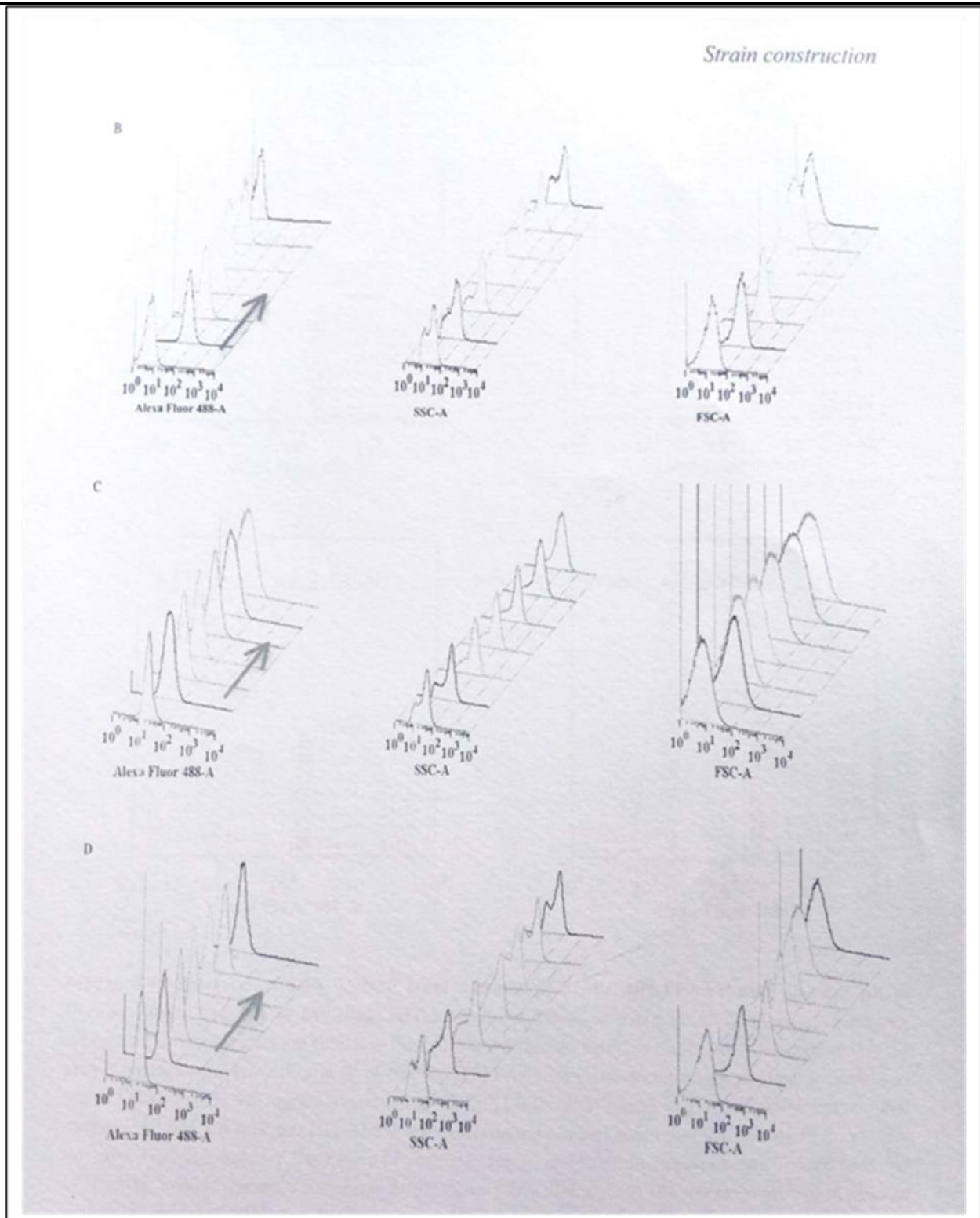
Figure 4.4 Fluorescence data derived from plasmid pGS20PrrnBGFPAr and plasmid pXl.0 PrrnBGFPmut3 in E. co/i MC1000. GFP intensity at 488nm is listed as Alexa Fluor, data on side scatter and forward scatter are shown in flow histogram format. Samples displayed order is shown by the arrow. Histograni overlay profile of pGS2OPrrnBGFPAAV in glucose containing minimal medium (A) and LB medium (B). Histogram overlay profile ofpXl.0 PrrnBGFPmut3 in glucose containing minimal medium (C) anJ LB medium (D). The Y-axis of histogram is cell count numher. X-axis is fluorescent intensity. CDF (Cumulative Distribution Function) plots (E and F) of the fluore:tce data derived from (A) to (D). the Y-axis is relative frequency from 0% to 100%. Subplots in (E) re growth in glucose contutning medium, left is for pGS2O plasmid, right panel for pX 1.0 plasmid. Subplots in (F) represent growth in LB m left is for pGS2O plasniid, right panel is for pXl.0 plasmtd.

A small shoulder appeared in the SSC and was more obvious in LB medium than in minimum medium, maybe because promoter rrnB P is more active by comparison with i,nB P promoter in rich mediurn and as so more complexity of cell activity is seen in rich medium where population tends to be fractionated easily. LB medium pros ides only a scant amount of carbohydrates, and small amounts of other utilizable carbon sources, whereas, for growth in glucose-containing medium, this limitation seems less enormous than in LB medium (Figure 4.4). It could also be a signature of the properties of the cell wall, nbosome content or amount of macromolecules per cell in the bacterial cell. This fraction of cells is not totally separated from the main body of the population and its size is reduced in mid exponential phase. When it turns to stationary phase, some cells remain in a different state distinct from the main population in SSC.

Degradable and stable GFP were both quickly synthesized in fast dividing cells and slowed down afterward because of the slower growth and less demanding of ribosomal proteins. The stepwise weakening of the destabilized GFP(AAV) signal intensity showed lower fluorescence concentration than GFPmut3 in late growing phase. Wild type GFPmut3 was rather stable signal with less induction and regression. Furthermore, it seems that the GFP induction correlates with forward scatter and side scatter. Similar increment and decrement trends have been found in FSC and GFP intensity.

The Overview of the whole population from the Cumulative Distribution Function plots indicates that the variation in LB medium is larger than in minimal medium, and more obvious in pGS2O than in pXl.O. Probably more variation of plasmid copy number occurred in pGS2O but not pXl.0. besides the nature of different versions of GFP. The use of destabilized GFP could minimize cellular signal accumulation, thus it is interesting to see if destabilized GFP can serve as a good indication of cell divisions in the fermentation context.

Plasmid stability

To test the plasmid stability of pGS2OPrrnBGFPAAV, a serial 100-fold dilutions of the overnight culture in fresh rich medium was carried out over 3 days at 37°C. 160 rpm. with no addition of antibiotics. fohowed by plating on LB plates. Then 768 colonies were restreaked on CHL-containing plates overnight. Three triplicates ve used. The number of cells without plasmids was calculated as the ratio of the non antibiotic resistant colonies to the total number of the streaked colonies. 41 out of 768 colonies did not grow on CHL plates and this corresponds to about 5% plasmid loss.

Conclusion

Growth reporters for E. co/i having GFPAAV expressed under ribosomal promoter was constructed and its signal showed a growth dependent manner as expected. Therefor, experiments with further environmental perturbations will be carried out to investigate the effect of environmental parameters on the population heterogeneity in the cultivation process. Meanwhile, plasmid based constructs will serve as copy number reporters in experiments where effects of environmental conditions on the plasmid copy number are concerned. As a complement of using reporter strains a number of fluorescent dyes targeting particular physiological features such as membrane integrity and metabolic activity are under consideration as well since these dyes would provide a full picture of the physiological state of the cell on single cell level from different angle. It has to be mentioned that the host strain used at this time for signal test was E. co/i MC 1000 and later all plasmids were transformed to E. co/i MG 1655 in the fermentation process due to its better growth behavior than MC 1000.

Acknowledgement

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5.1 Manuscript 1

Design of growth-dependent biosensors based on destabilized GFP for the detection of physiological behavior of Escherichia coli in heterogeneous bioreactors

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Abstract

In this work, we present the design and characterization of Green Fluorescent Protein (GFP) based reporter systems designed to describe cellular activity in "complex," heterogeneous bioreactors. The reporter

systems consist of Escherichia coli strains carrying growth dependent promoters fused to genes expressing stable and unstable variants of GFP, respectively. The response of Eschcrichia coli cells to transient exposure to glucose was studied in a two- compartment scale down bioreactor (SDR) consisting of a well-stirred tank reactor (STR) connected to a plug-flow reactor (PFR). Such a SDR system is employed to mimic the situation of high glucose concentration and oxygen limitation that often encountered in large-scale, fed-batch bioreactors and the response of E. coli was simulated by continuously pumping microbial cells from STR to the PFR. We found that repeated addition of concentrated glucose pulses with varied frequency at the entrance of the PFR had consequenc on strain physiological behavior. The GFP expressions were significantly marked after 10 h of cultivation in STR (control reactor) and SDR, whereas, growth rates were rather similar. Additional experiments in chemostat with programmed glucose perturbation suggested that the activities of the promoters were linked with the substrate limitation signal. Taken together with immunoblot analysis. we suppose protein leakage is responsible for the overexpression of/Is and the related promoters, such as rrnB in this case study. but additional works are required in order to confirm this relationship. This investigation is useful for a better understanding of the fast dynamic phenomena occurring in heterogeneous large-scale bioreactors.

Keywords: Destabilized GFP. Scale down, Heterogeneity, Escherichia coil

Introduction

Escherichia co/i is a well-studied bacterium, which is able to direct its metabolic pathways depending on the type of substrate present in the growth medium. Whenever growth conditions are not optimal, growth is challenged by stress, i.e., by products synthesis (short term response) and genetic reprogramming directing cells to a dormant state (long-term response, such as the general stress response governed by the sigma factor (rpoS) in the case of E. co/i). Physiological readjustments to environmental stresses involve multilayers of genes and proteins and are known as the cell stringent response.

In industrial bioprocesses controlled cultivation of microorganisms is essential in order to maintain production efficiency and quality. However, mixing inefficiency inside the reactor both spatially and temporally often leads to conditions in which substrate concentrations and/or physical parameters oscillates, continuously exposing the cells to gradients of for instance nutrients, oxygen and pH. This might lead to heterogeneities within the cellular population in a seeming homogeneous bioreactor where individuals behave differently according to the conditions experienced in their surroundings. Heterogeneities can be minimized but not avoided in large-scale bioreactors. particularly when operating under the fed batch mode. Among others, oxygen limitations and substrate gradients are particularly obvious in fed- batch cu1tivations, since a highly concentrated substrate is fed to the top surface of the culture through a narrow zone to avoid a significant increase in volume. Consequently, inhomogeneity is generated while cell circulating throughout the 22 m reactor. Cell segregation is further magnified by reactor enlargement as a result of deficient mixing. which leads to zones with diverse environmental conditions. The glucose gradients generated by improper mixing in this high density fermenter. a the main source of failure during the cultivation scaling up, were shown te be responsible of inducing four stress sensitive genes and negatively affecting heterologous protein production, product yields and cell viability. To quantify metabolic responses to nutrient perturbations,

Schweder et al. summarized E. co/i genes that respond to nutrient limitation, environmental stress, or those genes indicating the physiological status of the bacteria. Such rapid stimulus-response experiments have been applied to Saccharomyces cerevisiae. However, a limited analysis on cell response can be derived from the studies mentioned above and still less is known about the influence of such inhomogeneity on the process performance."

One attractive way of gaining information about potential heterogeneities within bioreactors is to use the microbial population itself as physiological tracer for cellular activity.' This can be done by using transcriptional fusions of inducible promoters and genes expressing fluorescent proteins (such as green fluorescent protein, GFP). GFP is an excellent signal tracer in biological research because of its bright and stable fluorophore, low toxicity, and ease of quantitative detection without disrupting the cell.' The first quantitative application of GFP to indicate the level of heterologous protein was provided by Albano et al.' Till now, GFP and its variants are used in many biological studies.' The wild type GFP with a high degree of stability accumulates to high levels that facilitate detection is suitable for analyzing the fluorescent signal over time. Unfortunately, it becomes unsuitable in the viability studies and the studies that require reporter protein turnover, such as in the analysis of transient gene expression in response to environmental signals. As the high degree of stability of the GFP protein (the fluorescent signal can last for over 24 hours' the non-active, non-viable or dormant bacterial cells containing stable GFP would continue to fluoresce or still retain GFP fluorescence in the absence of promoter gene expression' and the fluorescence would extend over many bacteria generations. it might pose a problem in experiments where transient and dynamic changes in gene expression is concerned. Therefore, to answer the question of how gene expression changes in individual cells as they respond to various environmental stimuli in real time, the use of a destabilized reporter system is preferred. Driven by this purpose, Andersen et al.' previously made destabilized variants of GFP by adding a C- terminal oligopeptide extension that targets GFP for de through an ssrA mediated degradation system. The newly created GFP is rapidly turned over by proteolysis and no fluorescence is accumulated. Dependmg on the last three amino acid of the C-tenninal tail, the protein variants' half-life is shortened to 110 mm, 60 mm and 40 mm for ASV-, AAV- and LVA/LAA-tag, respectively.' Since then, unstable GFP has enabled precise inspection in enormous studies, such as, monitoring nutrient availability and quantifying stress response of E. co GFP derivatives with a broader range of stabilities have been extended to yeast to detect cell cycle 60 dependent gene expression. The reported GFP half-life is as short as 30 mm in bacteria and yeast and CFP protein is as short as 5 mm in yeast.

Ordinary bioprocess monitoring often relies on population-based measurements of cell viability and yield production, which is unable to reflect the heterogeneity among individual cells within a population. A number of methods, such as flow cytometry and fluorescence microscopy have been applied to discover and monitor cell-to-cell differences, cellular mechanisms and regulatory circuits on the single-cell level. In front of other techniques, like RT-qPCR involving mRNA and fluorescence microscopy, flow cytometry has been extensively used to study the phenotypic diversity of cells used in industrial process. Flow cytometry can be used to analyze thousands of cells per second without the need of processing samples, although it provides a snapshot of gene expression at given time point.

In this study, each growth reporter strain contains a vector that was created by fusing g/ (stable and destabilized variant) to either the E. co/i promoter rrnBP]P2 or Jis. Therefore, within cells containing the reporter vector, any changes in fluorescent signal from the unstable GFP protein is seen as an indicative of promoter activity. rRNA synthesis is the first to respond to the nutrient up shift or down shift of the medium in order to meet the demand for protein synthesis and provide maximal protection for long term survival. It is known that regulation of rRNA synthesis is responsive to environmental and nutritional signals and inversely reacts to rRNA gene dosage. rRNA transcription is the rate-limiting step in ribosome synthesis and is controlled primarily at the level of initiation from rRNA promoters in E. co/i. The rRNA promoters are among the strongest in E. co/i and is accounting for distribution of the majority of RNA polymerase during rapid growth. Changes in nutrient availability result in almost immediate changes in rRNA promoter activity. The rrnBPlP2 promoter is sensitive to the concentrations of two small molecules, the initiating NTP (iNTP) and guanosine 5' -diphosphate 1' -diphosphate (ppGpp).'° FIS is a small nucleoid-associated protein involved in many cellular processes, including the activation of rRNA synthesis, and the expression of FIS has certain physiological effects on E. co/i, suggesting that FIS plays a global role in the adaption of E. co/i to changing environmental and nutritional conditions. For rrnBPl promoter most of the activation by F IS is due to the three FIS-binding sites located upstream and FIS acts as a transcription factor that trans activates the rrnB promoter (Figure 1). In addition to its role in recruiting RNA polymerase (RNAP) to the rrnBPJ, FIS may also affect the transcription initiation after the recruitment. The FIS expression pattern is quite similar to that of rRNA, and expression of the us promoter is regulated under the same conditions as rRNA promoters, as the Jis promoter responds to the same signals of nutrient availability that regulate rRNA promoters (e.g., iNTP concentration and ppGpp). So, FIS facilities the expression of rRNA when sensing the cell demands for protein synthesis. In other words, the expression of fis promoter is regulated by growth and in response to nutntional upshift. According to Huijun Zhi et al., this promoter is activated depending on the cellular nutritional status. Fis is highly expressed in the very early log phase of rapidly growing cells whereafter the promoter is switched off in late log phase and stationary phase. Fis expression is furthermore regulated FiS itself (feedback control) and the level of DNA supercoiling that couples to changes in cellular physiology.

The effects of environmental gradients and their exposure influence on cells can be studied.at the laboratory scale using scale-down experimental approaches. The most commonly used setup is a two-compartment scale down bioreactor (SDR) consisting of a well-stirred tank reactor (STR) compartment cormected to plug-flow reactor (PFR) compartment. Here in order to test their potential use as physiological tracer for process perturbation, the destabilized GFP reporter strains were tested both in lab- scale bioreactors and in scale-down bioreactor. This last configuration comprises a lab-scale stirred bioreactor connected to a tubular loop and is generally denominated SDR. The cultivation medium is continuously re-circulated into this recycle ioop in which glucose is injected. By this way glucose concentration gradient can be generated and microbial cells are stochastically exposed to environmental fluctuations such as those met in large-scale bioreactor. Unexpectedly, our growth-dependent biosensors have exhibited a continuous increase of GFP synthesis when cultivated in well-mixed fed-batch bioreactors, vherea this phenomenon was not observed in the SDR. Additional experiments conducted in chemostat and perturbed chemostat have confirmed the results. Analysis of supernatants has revealed that this phenomenon could be linked with protein leakage.
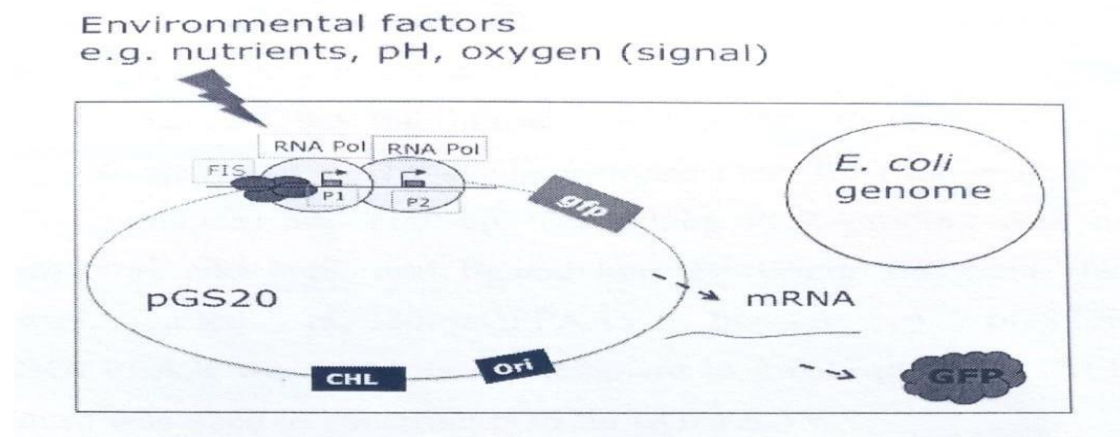
Figure 1. Plasmid pGS2O is used as the common backbone for constructing reporters in this study. Fis protein has three binding sites in front of rrnB P1 promoter. The physical location of rrnB P1P2 promoter and gf gene is illustrated. Alternatively, Jis promoter was used to replace rrnB P1P2 promoter to construct pGS2OfisGFPAAV.

Material and Methods Construction of reporter strains

Plasmid pGS2OPrniBGFPAAV and pGS2OPrmBGFPLAA: The gtj(AAV) gene, encoding the unstable GFP variant GFPAAV with a reported short half-life around 60 mm in E. co was PCR amplified from a plasmid carrying gfpmut3 by using primers GFP-up and GFP(AAV). The 760bp PCR product was digested with iVa'eI and sgrDl and subsequently inserted into the same sites of a low copy number plasmid backbone pGS2OPrrnB (colEl replication origin, 10 to 15 copies/cell. chioramphenicol resistant marker. 25 ig/ml) (Epoch Biolabs inc.. uS), which is canying the E. co/i ribosomal promoter rrnBPlP2. Thus. the resulting plasmid. pGS2OPrrnBGFPA;\V, expresses GFPAAV under the control of the nuB promoter. This plasmid furthermore contains a strong ITOB T transcription terminator to prevent readthrough from he promoter. The gj gene, encoding ihe unstable GFP variant GFPLAA. was reported to have short half-life around 40 mm in E. co/i. The primer pair used for amplifying gf gene is GFP-up and GFP LAA). Except the PCR primer, all other construction steps were the same as constructing pGS2OPrrnBGFPAAV.

Plasmid pGS2OFisGFPAAV and pGS2OFisGFPLAA: pGS2OPrrnBAAV was digested with Not! and NdeI in order to replace the rrnB promoter region with the E. co/i Jls promoter that encodes a DNA binding protein, FIS. The Jls promoter was amplified from the E. co/i chromosome by using primers FisP-SD (the primer contains an optimized SD sequence) and FisP-up. The 300bp PCR product was subsequenfly digested with Not! and NdeI and ligated into the vector fragment. The resulting plasmid was called pGS2OFisGFPAAV. Insteadof pGS2OPrrnBAAV, pGS2OPrrnBGFPLAA was used as the template to create pGS2OFisGFPLAA. The same procedure was used to construct pGS2OFisGFPAAV.

Plasmid pGS2OPrrnBGFPmut3: A NotI-Ndel fragment containing the PrrnB promoter from plasmid pGS2OPrrnBGFPAAV was cloned into the same sites of plasmid pGS2OPA lO4O3GFPmut3 thereby replacing the PA 10403 promoter.

The generated plasmids were finally transformed individually into E. co/i vildtype strain MG1655 by electroporation using a Bio-Rad Micropulser according to the manufacturer's instructions. Plasmid

verification was done by enzyme restriction and sequencing. Primers for plasmid construction are shown in Table 1.

Table. I Primers used in this study

Primer	Sequence (Relevant restriction sites are underlined) Primer
GFP-up	5'- ATATACATATGCGCAAAGGTGAAGAACTGTTTAC
	Ndel
GFP(AAV)	5'CTCTCCGTCCGACGATTAAACTGCTGCAGCGTAGTTTTCGTCGTTTGCTGC
	SgrDl
	AGGCCTTTTGTACAGTTCATCCATGCCG
	5'-CTCTCCGTCGACGATTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGCTGC
GFP (LAA)	SgrDl
	AGGCCTTTTGTACAGTTCATCCATGCCG
	5'-CCCCCATATGTAAATCCTCCTTATTGAAATGACCATACTGTGACTGCA
FisP-SD	Ndel
	AGGC
FisP-up	5'-CCCCCGCGGCCGCCTCAAAAGGACGTAAATTTGCCG
	Natl

Medium composition, inocu and bioreactor operating modes

The defined minimal medium (MM) used in this stud (g/L): K 14.6. NaH 3.6; Na2SO 2; (NH4) 2.47, NH 0.5. (NH 1, glucose 5, thiamine 0.01. Additionally, 3 milL of trace solution. 3 milL of a FeCl solution (16.7 giL). 3 ml/L of an LDTA solution (20.1 giL) and 2 ml/L of a MgSO solution (120 g/L) are supplemented. The trace solution contains (in g/L): CoCl 0.74, ZnSO 0.18, MnSO 0.1, CuSO 0.1, CoSO 0.21.

Working seeds (2 ml) of the strain were maintained at -80°C in MM with 40% of glycerol. A pre-cultivation step was performed in 100 ml of the mentioned medium (with glucose 10 gIL as an exception) in a baffled shake flask under orbital shaking at 37°C, 160 rpm. Cell growth was followed by optical density (OD) measurement at

600 nm. For cell dry weight measurement, the filtered and washed cultivation broth were dried during 24 hours at 105°C, cooled down in a desiccators and afterwards weighted on an analytical balance. Glucose concentration was measured with an electro-enzymatic system YSI (model 2700). The lab-scale stirred bioreactor (Biostat B-Twin, Sartorius) used in this study was configured with a standard Rushton turbine, and as described in detail elsewhere, the reactor is equipped with controls for dissolved oxygen concentration. pH, temperature. and aeration rate (remote control unit by the MFCS/win 3.0 software).

High c experiments were carried out in either the classical stilTed vessel (total volume: 3 L; initial working volume: 1 L: final working volume: 1.5 L; reference reactor configuration) or the stilTed vessel connected to a 12-meter recycle loop (silicon pipe) to generate a non-well mixed condition (scale down reactor configuration). This silicon pipe (diameter 0.005 in, length 12 in) leads to a mean residence time of 79.8 s (as measured in a previous study reproducing heterogeneities expected in large bioreactors. Dissolved oxygen concentration was monitored directly in the recycle loop by non-invasive optical sensors (flowthourgh cells — Presens). Medium was circulated between the stirred reactor and the recycle loop by a

peristaltic pump (Watson \Iarlo\\ 323) of 80 rpm, with the glucose feeding solution (500 ml 400 g/L glucose in minimum medium) at the inlet of the recycle loop to generate a substrate gr During the experiments, pH was maintained at 6.95 (regulation by ammonia and phosphoric acid) and temperature at 37°C. For reference and scale down reactor. initial agitation speed and aeration rate was set at 800 rpm. I vvm and 800 rpm, 0.5 vvm, respecti\'ely. Once feeding started, the agitation rate and aeration rate were progressively achieved to 1300 rpm and 2 vvm. Feeding control was based on dissolved oxygen level (setpoint fixed at 30% above saturation) in order to prevent oxygen limitation. This control was setup on the basis of a PID regulation previously considered. Samples were collected from the STR compartment every 1.5 hours. A further addition of MgSO and FeCl solution was added to the culture in the beginning and middle of the fed batch process (at an optical density of 50) in order to avoid problems associated with precipitation. All the fed-batch fermentations started with batch cultures, and a dissolved oxygen control based feeding was adopted when initial glucose was depleted.

Oscillation experiments were performed in chemostat mode at a chosen dilution rate of 0.14 h' and the agitation speed and aeration rate was set at 800 rpm, 0.5 vvm. Chemostat cultivation was preceded by a batch phase, which was carried out on a medium identical to the feed medium. Before starting medium feeding, cells were starved for 1 hour after glucose depletion at the end of batch phase, which was indicated by a steep rise of the dissolved oxygen, in order to see the impact of glucose pulse at the level of gfp synthesis. After reaching the steady state, perturbations were made by adding a glucose solution of 30 g/L (made up in distilled water) to the reactof following a switching profile controlled by a dedicated Matlab code. The ON OFF sequence was 100/900 s with an intermittently stopped flow rate of 0.08 ml per second. The time interval between two pulses was predetermined by the glucose consumption speed in the current reactor setting based on the DO response. DO was measured, but not controlled, and is only used to verify the glucose depletion in batch mode and aerobic conditions were maintained at all times in the chemostat. Samples were taken every 30 mm after the glucose feed start.

Antibiotics were added in the medium to prevent plasmid loss and the development of mutants and contamination during the cultivation. Foam was disrupted by addition of antifoam agent- polypropylene glycol (PPG) in demand. Independent cultivations were performed in duplicates.

Flow eytometry

The analysis of GFP expression level was performed with a FACscan flow cytometer (Becton Dickinson). Samples were divided in two subsamples, the untreated subsample for GFP analysis and the other for P1 staining at room temperature for 10 mm. Since we used a destabilized variant of GFP with a reported half-life of 1 hour, sampling was a critical step. In order to avoid hydrolysis of GFP by intracellular proteases between sampling and analysis, samples were diluted twice in a 30% glycerol solution and directly stored at -80°C. This process was validated by comparison with fresh samples analyzed directly by flow cytometry. For each measurement, 30,000 cell events were analyzed for gfp gene evolution with the excitation and emission wavelength at 488 nm and 530 nm. The FSC, FL1, FL2 and FL3 channels are logarithmically amplified with settings of FSC E00, FLI 500, FL2 420, FL3 420. FSC and SSC channel can be used to determine cell size and shape respectively. A threshold of 52 was set upon the SSC channel. Propidium iodide (PT) stained samples were passed through flow with setup of FL 1 500, FL2 420, FL3 520

to check cell membrane pern The results were evaluated with Flowjo (version 7.6.1) and represented as specific GFP intensity, which corresponds to GFP intensity per cell based on analyzing 30,000 cells with FACS.

Total protein determination and supernatant fluorescence measurement

The total amount of protein was measured with Folin Lowry rnethod. Samples withdrawn from bioreactor were centrifuged at 12000 rpm for 3 mm and filtered on 0.2 urn cellulose membrane in order to remove the cells. Fluorescence of the supernatant (samples of 200 iL on 96 wells black microtiter plate) was analyzed by spectrofluorirnetry (Victor 3 V Wallac, Perkin Elmer) and shown in arbitrary units, AU. Proteins from 7 iL of the supematant were separated on 30% polyacrylarnide gels (Biorad) by standard SDS-PAGE procedures. Immunoblot was performed to etect the band corresponding to GFP. Detailed information can be found in one of our previous work.

Mathematical modeling of the dynamics of GFP synthesis

A dynamic mathematical model was set up in order to predict GFP synthesis in batch and in continuous bioreactors. In order to simulate GFP synthesis, the following equation was used:

With .t, being the growth rate (h'), GFP the concentration of GFP (arbitrary unit) and rdecayaav, the decay rate constant of the gfpAA V (h The generation term in this equation is proportional to

l.t since this kind of ribosomal promoter is activated in a way responding to the so-called 'ji squared' rule and the synthesis of rRNA in a cell is proportional to the square of the growth rate of the culture. The value of t is driven by the bioreactor-operating mode. For the batch mode, the following ODEs were considered:

$$\frac{dX}{dt} = \mu_{max}.\frac{S}{K_S+S}.X = r_X.X \quad \text{(Equation 2)}$$

$$\frac{dS}{dt} = -\frac{r_X}{Y_{X/S}} \quad \text{(Equation 3)}$$

With X, S and CL being respectively the biomass, substrate and dissolved oxygen concentration (gIL or mg/L). Ks being the affinity constant for substrate. Yx being the coefficient yield of biomass from glucose (g/g). l.tmax is the maximum growth rate (h The equations were simulated by using the 'ode23s' routine available in MatLab R2009b (The MathWorks, Inc., Natick, MA, USA). Most of the simulation parameters were adjusted on the basis of the dynamics observed experimentally in our cultivation conditions, i.e. microbial growth and substrate consumption curve. All these parameters were kept constant during the simulation while the parameter r was varied in order to show the effect of the GFP stability on the reporter behavior.

Results and Discussion

Predictive modeling and experimental validation of the behavior of GFP biosensor in batch bioreactor

Growth rate-dependent control in bacteria involves regulation at the levels of transcription and translation, and an increase in growth rate is asso:iaied with an icre in cell size and number of ribosomes. In this study. reportc strains based on the expression of destabilized GFP under the control of either rrnB Y1P2 promoter orfis were employed to illustrate the dynamic growth response of bacicria population in heterogeneous bioreactors. Those two promoters may provide complementary nnal in responding to nutrient

availability since they have similar expression pattern. The GFP expression level is seen as the function of promoter strength. We 'istructed several different plasmids, which were later introduced individually into

E. coli strain MG 1655. Due to the rapid turnover of destabilized GFPLAA (half life is about 40 mm and the low copy number of reporter protein, the majority of cells exhibited very low fluorescence that is not suitable in this study for detection using flow cytometry (data not shown). Therefore, we decide to continue the work with GFPAAV that has a higher fluorescence.

To predict the behavior of the constructed biosensors in function of the bioreactor operation mode a dynamic mass balance model was used. GFP synthesis was modeled by an ordinary differential equation comprising three terms, i.e. a production term responding to the I' squared rule and two distinct consumption terms taking into account GFP dilution by cell division and the GFP decay under the action of intracellular proteases. This last term is very important since most microbial GFP biosensors are based on GFP variants exhibiting a very high stability with half-life exceeding 24 h. This intrinsic stability limits the use of the GFP reporter system for the detection of microbial stress in bioreactor operations, since physiological processes involving smaller characteristic times can be hidden by this property of the reporter molecule. Several simulations were made and a special attention was given on the impact of the stability of GFP varies from 0.1 to 10 h. Figure 2A shows the GFP signal cannot be directly correlated to the growth rate in the late exponential phase of a batch culture for the stability time exceeding 2 h by considering the intrinsic stability of the reporter protein.

As shown in Figure 2B, dynamics of the irnB:.gf,bAAVreporter system in cells grown in a batch bioreactor can be modeled with a stability half-life of GFPAAV between 45 and 60 mm, which is close to the previously reported value for this parameter. The data shows that both the stable and the desrab reporter systems respond quite well to the ji-squared rule (Equation 1, Figure 2C) in batch cultivation as expected in early exponential phase, however, GFPmut3 is more stable than GFPAAV and not follows the ti—squared rule hen growth is s ced down (Figure 2B and 2C). The simulations point out the fact that the stahilit\ of GFP is of primary importance br the quality of detection of variation in growth condF by the biosensor.
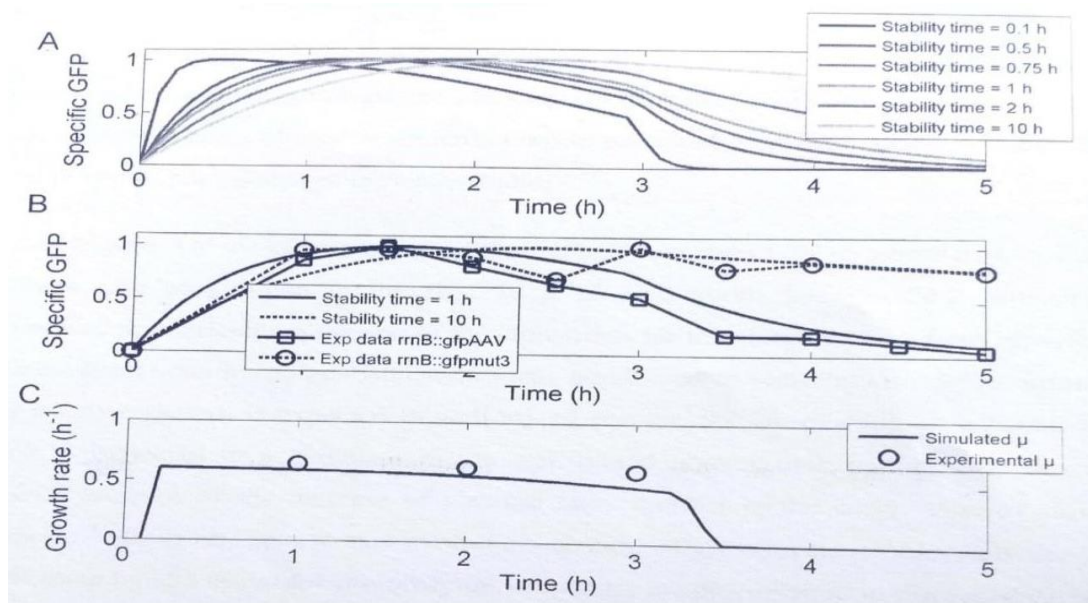

Figure 2. Comparison of simulated and experimental data for specific GFP dynamics in batch bioreactor. A: Several simulations were estimated by varying the stability time of the GFP from 0.1 to

10 h. B: Experimental data were extracted from a hatch culture of E. coli carrying either an rrnB::gfp.4A or an rrnB::gfpinut3 reporter system. GFP intensities were normalized by the maximum fluorescence of each sample. C: Experimental and simulated growth rate values.

Scale-down effect on growth dependent GFP biosensors: destabilized variant exhibits an unexpected behavior in homogeneous conditions

The objecti\e of this work s to determine whether growth-dependent GFP biosensors are able to detect process- perturbations. A set of fed-hatch culti- anons was used in order to e aluate he expression profile of our GFP biosensors n process- related stress conditions. 1- this purpose two kinds of promoters vere u namely. the ribosornal promoter r,i and thefis promoter. The fed-batch operatllig mode is particularly relevant since is the one most frequently used at the industr le and is also known to prom-te concentration gradient at the industriai scale. A corresponding scale-down system, that is, a well-mixed reactor equipped with a recirculation loop inducing the appearance of local heterogeneities, was also employed for each reporter strain. The two-compartment scale-down reactor (SDR) allows reproduction of mixing imperfections expected at large-scale and was used for that purpose in a number of previous studies.

From Figure 3A we can see that GFP synthesis rate seems to be correlated to the growth rate progression in the first 10 h of cultivation. Specific GFP intensity increased dramatically at the end of the cultivation for the cultures carried out in well- mixed conditions by comparison with those conducted in scale-down configuration. As a better growth is expected in well-mixed reactor, therefore, a higher amount of GFP is expected to accumulate in the well-mixed reactor than in. the scale down reactor because of the increase of plasmid copy number in the early stage of slow grov h. However, growth rate evolutions (Figure 3B) were very similar between a well mixed and a scale-down bioreactor, indicating the discrepancy in fluorescence of the Iwo reactors was not caused by the variation of plasniid copy number but additiona1 mechanism accounts for this process. The other reason to explain GFP accumulation might be that more cells lost their capability of hydrolyzing produced protein in the well-mixed reactor. No fluorescent diJ'crence was observed in the case of ti rrnB:.gfprnut3 biosensor, suggesting that the destabilized version of the biosensor is more suited to point out differences between well-mixed and scale-down modes. Glucose was the growth-limiting factor during the fed-batch phase and the amount of glucose varied between 0.01 and 0.25 g in the mixed compartment of the SDR. which is in accordance with our earlier reported data in the same bioreactor oper condition. Microbial cells in this kind of reactor are indeed exposed to stoci astic em ironmental fluctuations mainly governed by the random passage bet en the well-mixed part and the tubular pL: t. so GFP fluorescence can be impured by dissolved oxygen deprivation and gluc fluctuat ons inside the tubular 1 Dissolved oxygen fiuctuation were observed experimentally in the SDRs and sho\\ a in Figure 4. Clearly we can see that microb cells were exposed to dissolved oxvean limitation when crossing the recycle loop. vhereas these effects on the GFP man. ation steps can be excluded by considering the relatively short residence time of the calls in the tubular loop of the SDR (around 70 by comparison with 6.5 mm for G FP nut3 maturation
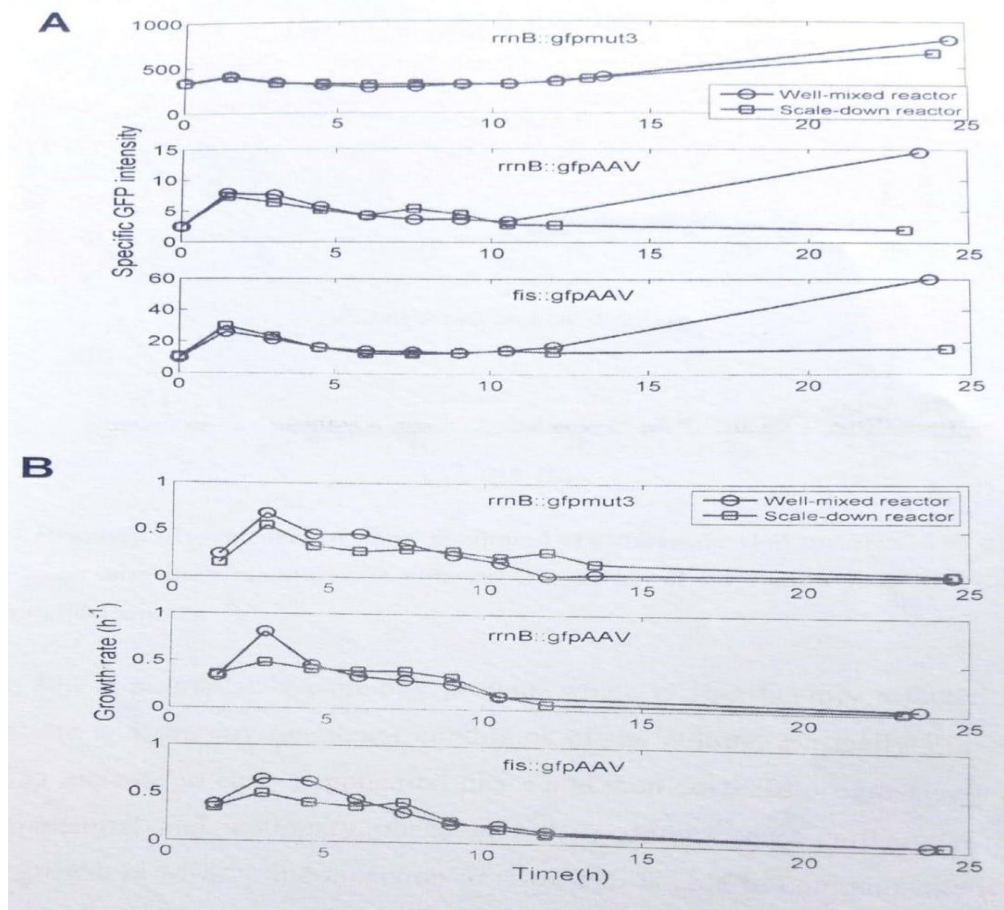
Figure 3. A: Evolution of specific GIP intensity and B: growth rate in well- mixed and scale down bioreactors operating in led-batch mode. The subplots from top to bottom panel cach represent E. co/i strain bearing rrnBgfpmut3, rrnBgfpAAV. or fisgfpAAV.

To confirm that the evolutic i of GFP a v mixed and non veli naxed ieactor became distinct at the end of e fed batch process, experiments using the tl.c::gt V biosensor were reproduced \-uh a higher sampling frequency of I Ii (Figure 5). Dynamic change of JIs acti\ .t is relatively vell documented in the literature and could contribute to the understanding of the molecular processes involved in our experimental conditions.
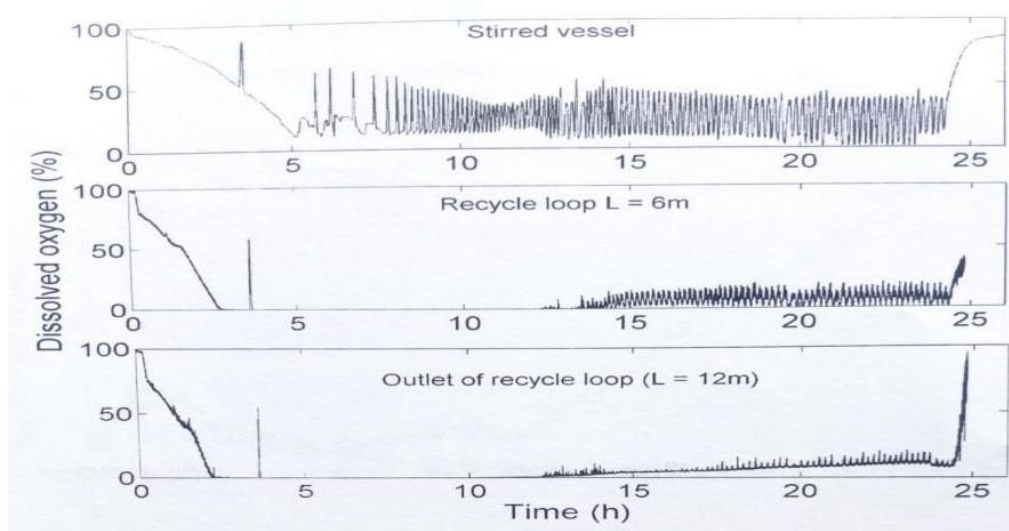

Figure 4 Dissolved oxygen concentrations monitored at different level of the SDR. The dissolved oxygen levels have been monitored at different lengths (L) of the recycle loop by using non invasive optical sensors.

Indeed, FIS is a small DNA-binding protein, which is significantly affected by the growth rate and the environmental conditions of the culture. Basically FIS level is known to increase in early exponential phase and then decrease progressively during late exponential and stanonarv phase in rich medium, or significantly reduced during growth in \4 In connection to rinB, FIS is able to communicate with the nutritional quality of the environment and is invol\ed in the regulation of ribosomes svnthesis. An important observation that was previously made is that FIS is overexpressed afler a nutrient upshift, for instance, when cells are rapidly transfelTed to a rich niedium. This observation matches the result obtained in well-mixed fed- batch reactor in this sndy. T overexpression of fis promoter was significantly marked after 10 11 of culturing tn current experiment, ho\\ ever, in our case a mineral salt medium was user: i : tout any addition of "rich" component such as amino acids. II k relatively difficu to assess this particular phv effect from experiments conducted in SDR, even though the condition in the SDR caused interesung deviations in the GFP evolution. Therefore additional experiments are needed in order to determine the source o excess expression. To this purpose, the dynamics of the fis and rnnB promoters will be confirmed in stabilized chemostat experiments and the results are described in the next section.
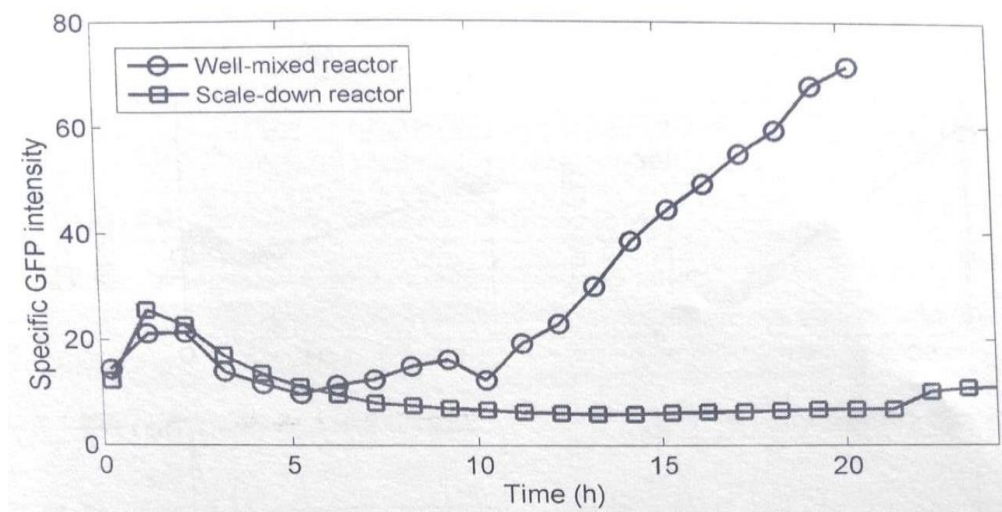

Figure 5. Evolution of the intracellular GFP intensity for fed-batch cultures carried out with the fis::gfpAA V biosensor in welt-mixed reactor and scale-down reactor. (Sampling frequency: I h).

Increasing activity of growth dependent promoters is confirmed in chemostat mode and is relieved when glucose oscillations are imposed to the system

The chemostat mode imposes a growth-limiting condition such as those experienced n prolonged fed-batch bioreactor. In chemostat cultivation cells are maintained in stabilized conditions (i.e. constant pH and dissolved oxygen). both the rrnB and the Yls biosersor exhibited the same excess expression pattern as that observed in well mixed fed-batch bioreactor (Figure 6). The GFP signal of bobi reporters increased to a le\ ci be\ ond the maximal level observed during the batch phase. This observation con drms the earlier results and suggests that the excess e' is linked with ul- trate limitation. Substrate limitation was partially rdieved in SDRs while

L cells crossing the tubular part where local substrate excess can be cn unte In order to simulate this kind of effect, repeated glucose pulses of fixed e were injected into the prior stabilized chemostat culture by using an abrupt O OFF DO-feed control. The time interval between two pulses was predetermined by the g consumption speed, that is. no glucose pulse was fed before the prior glucose was completely consumed.

During the experimental feeding process glucose concentrations were monitored to insure that no glucose accumulation occurred in the oscillating chemostat.
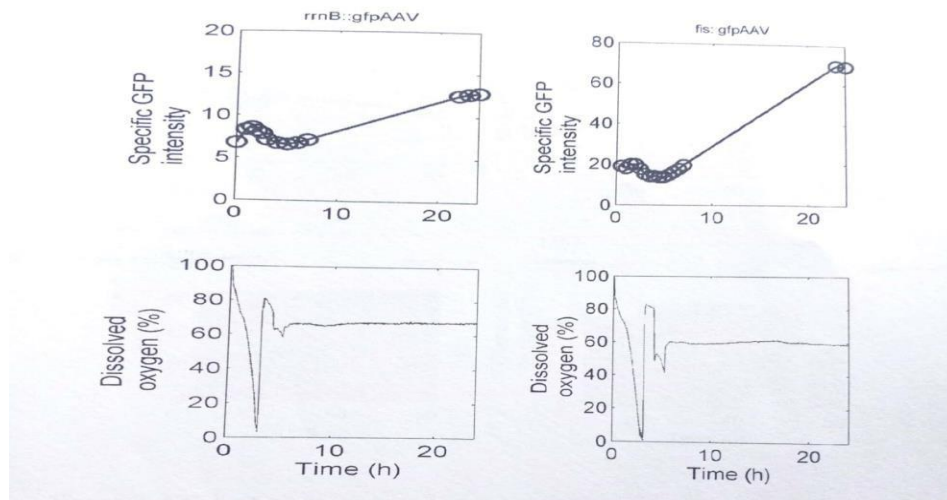

Figure 6. Evolution of the GFP signal from rrnB::gfpAAV(left) and fis::gfpAAV (right) biosensors cultivated in chemostat mode (D=O.14 h'). The steep rise of the dissolved oxygen indicates the end of batch phase and glucose depletion.

The result showed that glucose concentration was maintained below 0.056 /L and a short increase of GFP signal in the beginning of feeding was observed followed by a arogressive reduction of the GFP signal of the rrnB and JIs biosensors after hours of oscillations Figure 7). Repeated glucose pulses of fixed frequency were speculated to relieve the carbon limitation signal and accordingly the GFP signal decreased. This observation :ogcther with the finding of fis and rrnB excess expression in ed-batch conditions ci)ntirms the fact that the reporter behavior are linked with the substrate limitation sienal. Our previous results showed that protein leakage is induced when are cultivated in fed-batch reactor or in chemostat mode.' This leakage of protein into the exiracellular medium could possibly explain the JIs and rrnB overexpression after a sudden upshift to 'rich' medium. To assess this hypothesis, protein leakage was investigated in our operating conditions.
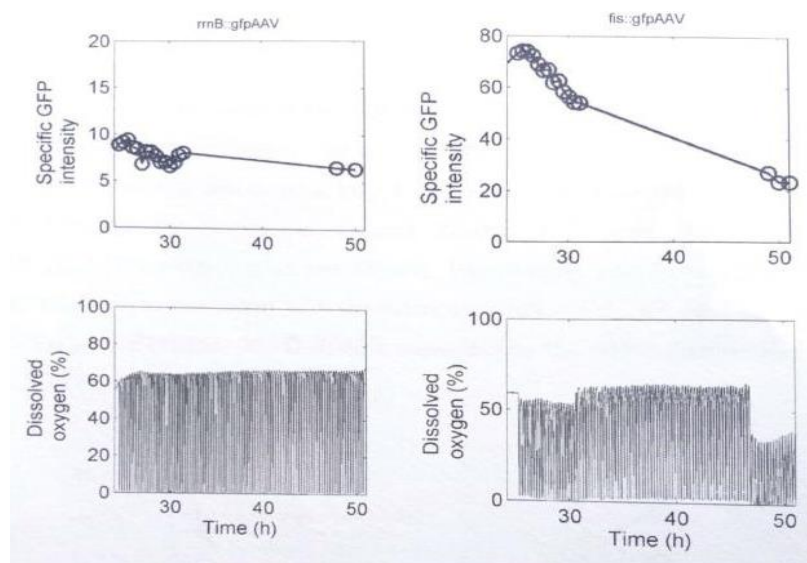

Figure 7. Effect of programmed glucose pulses on the fluorescence intensity of rrnB::gfpAA V (left) and fis::gfpAA V (right) biosensors cultivated in chemostat mode at a dilution rate of D =0.14 h'.

The effect of long-term exposure to nutrient limitation could possibly be explained by protein leakage

Microbial cells can alter their outer membrane structure and protein composition to facilitate substrate uptake under various growth conditions and glucose-feeding rate in continuous or fed- batch cultivations. Altered membrane structure might in turn facilitate protein leakage. Protein leakage is found to be twice as high in chemostat cultivation compared to the constant leakage level in fed-batch. Mechanisms associated with the increase of membrane permeability are associated with the up- regulation of porins and sugar transporters, as well as changes at the level of composition. We therefore decided to examine membrane permeability and protein leakage in our bioreactor set-up. This was achieved by looking into three parameters: the total protein content in the supernatant (Figure 8), the presence of

GFP in the supematant (Figure 9) and the membrane permeability (Figure 10). We found that the protein concentration was significantly higher in the supematant of well-mixed bioreactors compared to SDRs (Figure 8). This result is consistent with above results showing that microbial cells are more susceptible to protein leakage under repeated high glucose zones in well-mixed bioreactors, where a higher frequency of glucose injection was encountered. Consistent with this, Figure 9 shows that the amount of GFP protein as well as the fluorescent signal were significantly higher in the supernatant coming from a well-mixed bioreactor compared to a SDR, both harboring the Jis: :gfpAA V reporter. Similar results were obtained with the rrnB: :gfpAA V construct (data not shown). Interestingly, total extracellular protein concentration are consistent with the induction profile of the fis::gfpAA V biosensor mention above (Figure 9C, D to be compared with the GFP induction profiles of JIs: :gfpAA V depicted at Figure 5).
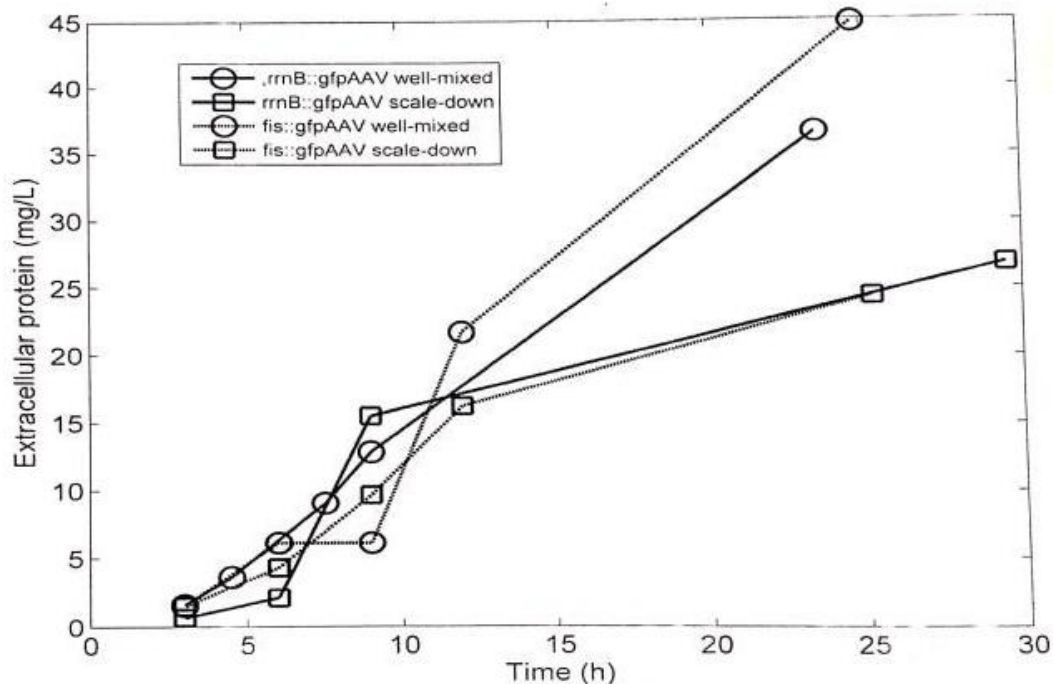

Figure 8 Comparative evolution of the total protein concentration of supernatant colIe from a vell mixed fed-batch bioreactor and from a scale down reactor.
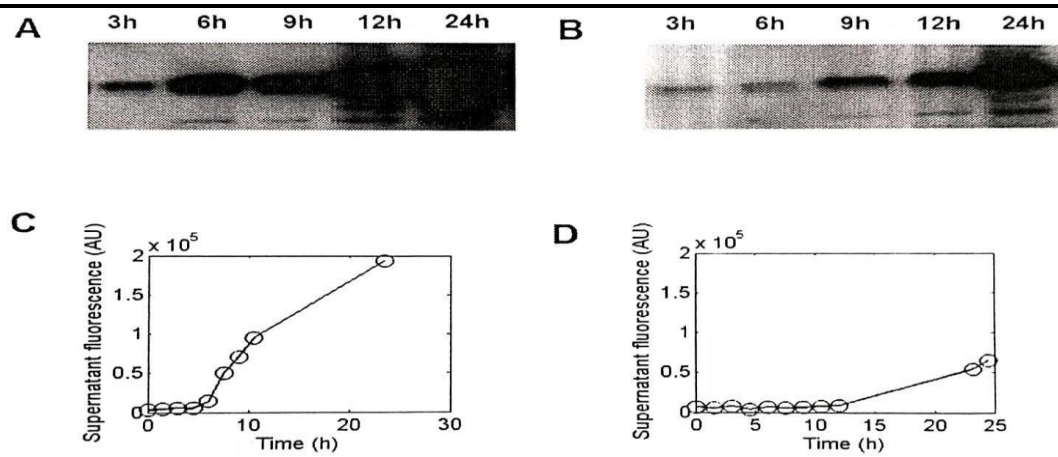
Figure 9. Representative immunoblot analysis of GFP coming from the supernatant of a culture carrying thefis reporter in a well-mixed (A) and a scale- down bioreactor (B). Evolution of the supernatant fluorescence (in arbitrary units, A.U.) of the fis reporter culture in a vell-mixed (C) and a scale-down bioreactor (D).
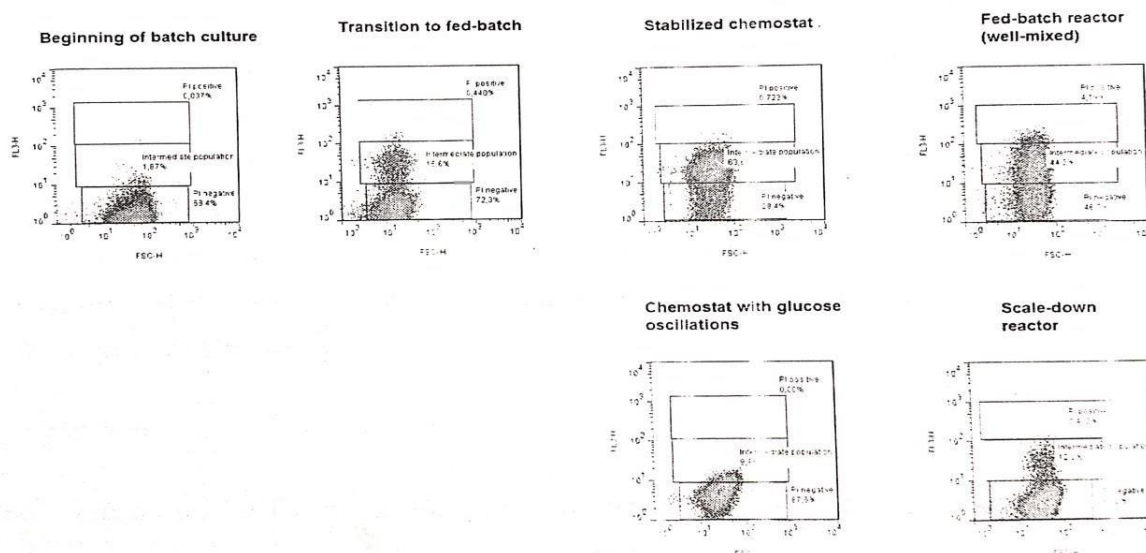
Figure 10. Susceptibility of the jis::gfpA4 V microbial population to P1 uptake in different operating conditions. Samples for the fed- batch reactor, well-mixed and scale-down reactor were taken at the end of the cultivation.

To assay membrane permeability, we used the dye exclusion test based on propidium iodide (P1) uptake for thejis reporter strain (similar observations were found with the rrnB reporter strain (data not shown)). In Figure 10, an intermediate subpopulation between P1 negative (intact membrane) and P1 positive (damaged membrane) cells are noticed in almost all the operating conditions. This subpopulation is particularly prominent in cultures grown in a stabilized chemostat and in well-mixed fed-batch bioreactors, but is almost absent in the cultures carried out either in chemostat with glucose oscillations or in SDR (Figure 10). It can be hypothesized that this population corresponds to cells with a permeabilized outer membrane leading to protein leakage and protein leakage in turn modifies the nature of the medium, thus this medium can no longer be considered as minimal medium in which proteins and amino acids are released to. Apart form this, the accumulation of amino acid from elevated amino acid synthesis pathway was stimulated by the glucose pulse. Regardless of the specific reason for medium enrichment, the appearance of the

permeabilized cell membrane represents a shift from minimal growth conditions to 'rich' growth conditions. This hypothesis is partially validated by the up-regulation of the tis promoter in stabilized chemostat and in well-mixed fed-batch reactor where protein leakage is significant, because JIs promoter is indeed known to be up-regulated when cells are transferred from minimal to rich medium (sce introduction). The potential release of proteins to the extracellular medium is intere since this phenomenon could possibly explain the long-term effect on expression of our gf reporters. it may also interpret that there is GFP accumulation in the vell-mixed reactor because of the loss capability of cells to degrade GFP protein, however. t can be argued that all ihese phenomena are linked in the context of this work.

Conclusion

The commonly utilized lTuoresc proteins such as (IFP can seve as reliable reporters in gene induction tudic. 1-lowever. their applnation is limted due to hc slow degradation rates. Rapid ch in gene transcription can be of interest wl they occur in responsc to physioiogical signals. Typically, fluorescent reporters tire used to monitor gene expression vnarnics at the translational level. TLe advantage of using destabilized reporter s evidcnt in comparison \\ith the stable GFI because if the GFP takes a long time to mature md degrade. then fluorescence wou stay and the reporter system would have 'memory' effect. To be a good temporal marker, the destabilized fluorescent protein is expected to have a quicker turn over, therefore, gene expression can be easily analyzed in living cells in nearly real time by using fast turn over reporter proteins. However, at meantime, the rapid degradation always problematically maintains a low fluorescence signal that is maybe hardly detectable. In this study two destabilized GFP biosensors were successfully constructed and used to estimate the performances between a well-mixed reactor and a non-well mixed reactor (SDR). Unexpectedly we found a progressive increase of GFP synthesis when biosensors were cultivated in well-mixed bioreactors by comparison with SDRs. This observation was made both for the rrnB: :gfpAA V and the fis. :gfpAA V reporters. This similarity between the behaviors of the two growth-dependent promoters can be attributed to the fact that they have similar expression pattern and Fis protein is known to act as an activator of the rrnB promoter. This phenomenon was confirmed in chemostat with and without controlled glucose oscillations, where increment of the GFP intensity followed by its decrease was found. Taken together with results from fed batch studies. it suggests that there may exist a relationship between the reporter behavior and the glucose limitation signal. Our previous findings have driven our attention on the fact that prolonged elucose limitation induces protein leakage.' Since JIs is overexpressed when cells are exposed to a nutrient upshift, such as the conditions being met in well-mixed reactors when proteins are released into the extracellular medium and it might be the reason of inducing GFP over expression since the medium was turned into 'rich' by the released amino acid or peptide. Analysis of supenlatant showed that the potennal protein leakage was significantly lower in s.ale-down conditions, demonstrating less protein release and lower gene overexpres in SDR. From a practical perspective, our results suggest that the difference : well mixed and SDR condnions could be easily detected in the earl\ stage of the cultivation. The results depiL ted at figure showed that the GFP proP le ex bited in the two cultl\ution conditions diverge fter 6 h of cultures. This divergence was correlated with major change' at the ph siological level, such as membrane permeability and release of proteins into the extracellular medium.

The rapid t results in less accumulation in cells leading to lower toxicity in the cells. Our Jestabilized GFP biosensors are thus very promising in order to detect mixing imperfections but also major physiological changes of industrial interest such as protein leakage. The potentialities of this technology would be only fully exploited if online tools are available in order to monitor fluorescence changes. For this purpose, the use of destabilized GFP biosensors in conjunction with online flow cytometers would be of great interest for the development of new single cell analytical tools."

The authors have declared no conflict of interest.

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5.2 Manuscript 2

A low-cost automated flow cytometry procedure for the characterization of microbial stress dynamics in bioreactors

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Abstract

Actual bioreactor performances rely mainly on the possibility to gain insight about process dynamics via on-line parameters and actual Process Analytical Technologies (PAT) recommendations are focused mainly on the increases of the on-line variables available for bioprocess documentation and control. In this context, flow cytometry (FC) has been automated in order to give access to the dynamics of single-cell physiological parameters in process-related conditions. This approach is not yet widespread applied both at the academic and the industrial level, probably because the technical challenges behind the automatization of FC. In this work, we propose a simple and low cost automated FC system allowing us to monitor two distinct cell physiology related parameters, i.e. the synthesis of a destabilized Green Fluorescent Protein (GFP) that under the regulation of the JIs promoter and propidium iodide (P1) uptake. The automated FC enables to compute these parameters at the single-cell level and at regular interval of 1 5 minutes. in addition, the mean to median ratio of these two parameters is a good indicator as a way to quantify the degree of population segregation

Keywords: Flow cytornetry, phenotypic heterogeneity, Escherichia coli, membrane permeability, green fluorescent protein

Introduction

Flow cytometry (F'C) is a very powerful tool in the follow up study of physiological properties of microbial cells in process-related conditions. The main advantage of this methodology is that simultaneous quantification of hundreds of thousands of individual cells per minute based on cell size, granularity and fluorescence properties in different channels provides loads of information at single cell level about the

phenotypic heterogeneity of a microbial population. This information is critical in a bioprocess improvement perspective since the appearance of unwanted phenotype can impair cultivation efficiency and product quality. Such phenomena has recently been pointed out during culture of recombinant Pichia pastoris with the appearance of a non-secreting phenotype. In the process analytical technology (PAT) framework, FC is proposed as an efficient on-line monitoring system for bioprocesses evaluation. The development of the on-line FC has been used for monitoring of recombinant protein synthesis. determination of mammalian cell viability, isolating robust microbial phenotype and monitoring water disinfection." However, the use of automated FC is still largely underexploited in front of its powerfulness in bioprocess optimization. Commercial systems, such as the Flowcytoprep (MSP corp, MN) device, are also available but are generally expensive.' In this work, we propose to use a bench BD AccurilM flow cytometer as a basis for the design of an automated FC that interfaces smoothly with automated bioreactor sampling systems. This apparatus is simple, easy to handle and leads to real-time, accurate data for process evaluation.' The presence of peristaltic pumps controlled by a microcontroller facilitates the interfacing of this apparatus to a bioreactor since no pressurization of the sample is needed to ensure the fluid displacement. Sample dilution and staining is carried out in the tubing between the FC and the bioreactor.

Spatial heterogeneity occurs during the scale-up of cultivation from laboratory to production volume due to imperfect mixing, consequently cellular heterogeneity is generated from the fact that each cell behaves more or less different from the other. even in the culture that with a homogenous nature. Therefore, averaged measurement of cell characteristics from samples of millions of cells cells has to be updated with single cell analysis, such as flow cytometric analysis, to picture the heterogeneous population structure at a single-cell as well as at a population level. Our previous study pointed out that the fis promoter is up regulated when cells are exposed to prolonged substrate limitation.' This phenomenon could be attributed to the release of extracellular components during the cultivation that can stimulate the fis activity, which was previously shown to be up-regulated when cells shifted from a minimum to a rich medium.' However, additional information about cell physiology is required in order to confirm this hypothesis. This automated FC system will be used in the follow up study of Escherichia co/i MG1655/pGS2OPfisGFPAAV fluorescent biosensor in the cultivation context, due to the fact that the current destabilized GFP variant exhibited a half-life less than one hour.' Distribution of cellular responses to glucose oscillation and the temporal evolution of a single cell by using fluorescent reporters will be recorded in a time dependent maimer.

Material and methods

Reporter strain, medium and pre-cultivation

Escherichia co/i MG 1655 bearing a pGS2O plasmid with a cassette ofJis::g gene and a chioramphenicol resistance gene was used in this work. Detail information about strain construction and pre-cultivation condition can be found in our prior study.' During the cultivation, 25 ig/mL of chlorarnphenicol (stock concentration:

50 mg/mI in 96% ethanol) was added to medium to keep the selection pressure.

Automated FC platform and sample analysis

The interfacing system between the bioreactor and the BD Accuri C6 has been performed on the basis of in-line mixing principle (figure 1A).
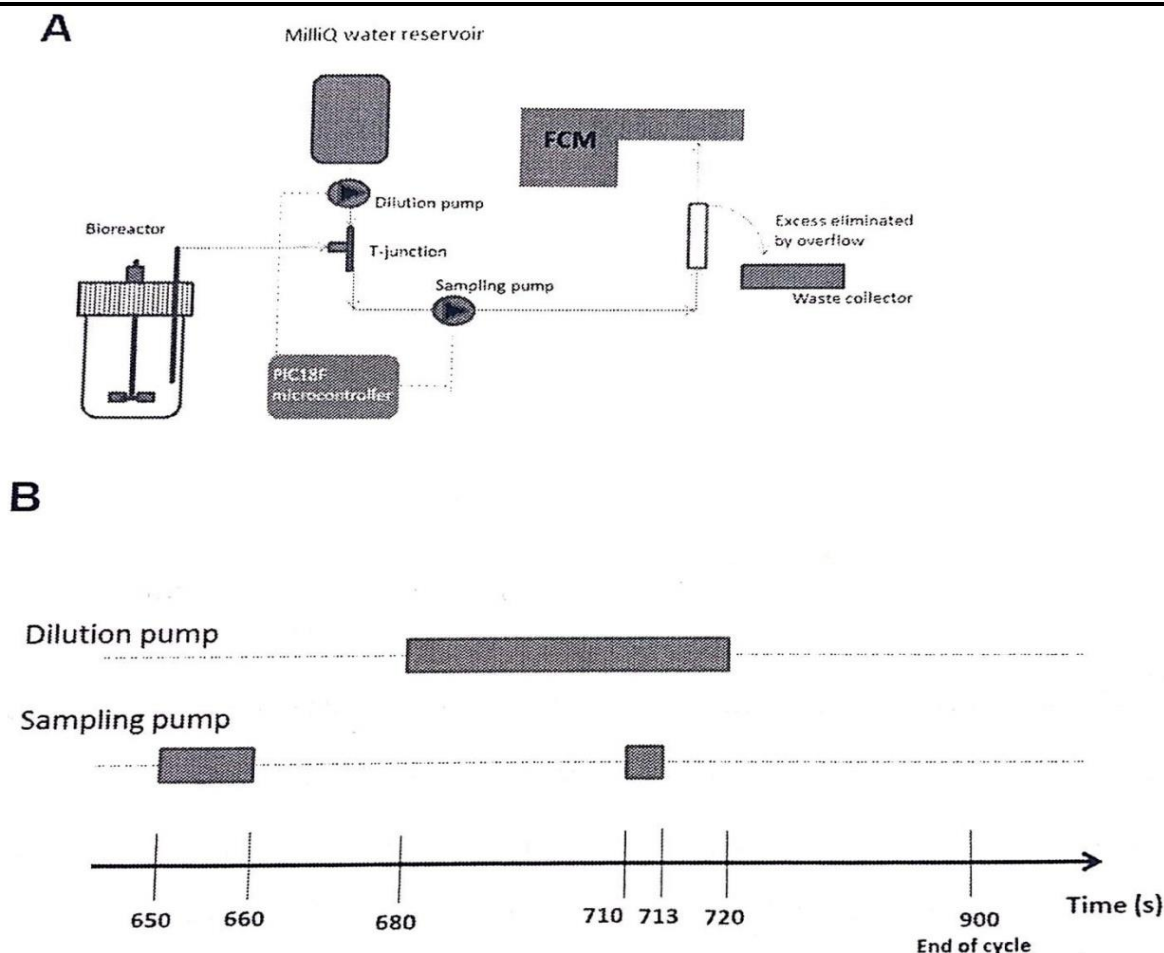
Figure 1. Flow platform is simplified as the interface between the bioreactor and the FC (A) and activation sequence of the dilution-and sampling pump every 15 mim (B). Sampling pump and the dilution pump are T-connected and operated by the microcontroller. The sample broth is overflown into the waste collection and the actual activation of both pumps with the 15 mm interval was simplified in the time scale of seconds.

A first peristaltic pump (Watson Marlow 040.DPID.N2R operating at a rate of 32 mL/min), as the sampling pump, is set to extract cell suspension (sample) directly out of the bioreactor and inject it in a flow of dilution water (milli Q water as the sheath fluid for Accuri C6). The dilution fluid is carried by a sequential peristaltic pump (Watson Marlow 040.DP1D.N2R operating at a rate of 32 mL/min), denoted as dilution pump, and mixed with the sampling line. In line mixing is achieved by a polypropylene T-mixer. Silicon pipes (internal diameter ID: 1 mm) are mainly used to build up the system, with an exception for the peristaltic pump where a long life Marprene tubing is used. All the connections are locked by Male Polypropylene Luer plug (ID: 1.6 mm). The activation or deactivation sequence of these two peristaltic pumps is controlled by a PIC18F microcontroller embedded in a full PICPLC16 control card provided by mikroElektronika supplier (the different sequences are illustrated at figure 1B).

This automated FC platform was preliminary adjusted to ensure the full cleaning of sampling lines to avoid cross-contamination between consecutive samples. A linear and efficient range of fluorescent analysis was found for samples with less than $3* 10$ events per mL (figure 1), therefore, before FC analyzing appropriate sample dilution was made and the dilution efficiency is validated (data not shown). Following inoculation, each sample at an interval of 15 mm is pumped through the FC analyzing station at a flow rate of 33 tL and approximately 200,000 events is thus recorded within 1 minute recording time. The recorded mean

and median fluorescence signal in the FL 1 and FL3 channel are corresponding to the GFP signal and the membrane permeability that measured by propidium iodide (P1) staining, respectively. Membrane permeability by P1 measurement was done by its direct addition into the milliQ reservoir at a concentration of 10 ig/mL. The staining procedure is validated and it proved that the incubation for 2 minutes is sufficient for appropriate P1 staining (data not shown). Processing and analysis of the flow cytometry raw data was performed using MatLab ® R20 lOb (The Math Works, Inc., Natick, MA, USA).
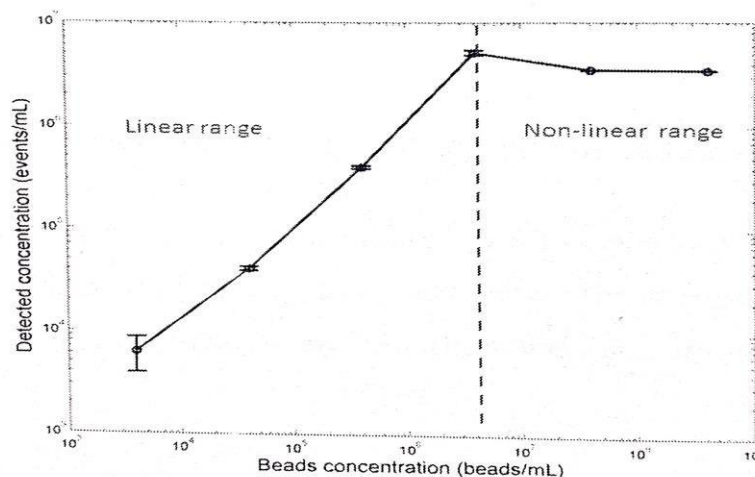
Figure 2. Determination of the linear range for fluorescent analysis using accuri C6 flow cytometer. Calibration was made by using 1 tm green fluorescent microsphere (Fluorosphere, Invitrogen) and the linear range of particals anJ fluorescence intensity can be found up to 3* 106 events per rnL.

Cultivation conditions

Chemostats were carried out in lab-scale stirred bioreactors (Biostat B-Twin, Sartorius with a total volume of 3 L; working volume of I L; mixing provided by a standard Rushton turbine) controlled by the remote control unit with the MFCS/win 3.0 software. During the experiments, pH was maintained at 6.9 (regulation by ammonia and phosphoric acid) and the culture was grown at 37°C and stirred at 1000 rpm with air flow rate at 1 L/min. Chemostat with a feeding solution containing 5 gIL of glucose solution (with the same minimal medium as previously described) at a dilution rate of 0.14 h'. Glucose oscillations were performed by pulsing 8 mL of a concentrated glucose solution (30 g/L made up in distilled water) in each 15 minutes cycle.

Results and discussion

Automated online sampling of the culture using flow cytometry provides high- resolution analysis for process monitoring. This platform consisting of sampling from bioreactor, sample preparation including staining with fluorescent dye P1, and sample injection into Accuri C6 flow cytometer, is a rapid online measurement of single cell properties. The application of this platform is extended in a biological study where heterogeneous population response and distribution was investigated over time by perturbing the steady state E. coli culture with a concentrated glucose pulse at growth rate of D=0.14 h'.

Follow-up investigation of theJis::gf by automated FC

In order to test the efficiency of the automated FC platform. validation test was carried out on the basis of our previous results. where the fis::g: V reporter sYstem is shown strongly sensitive to substrate limitation and fluctuation
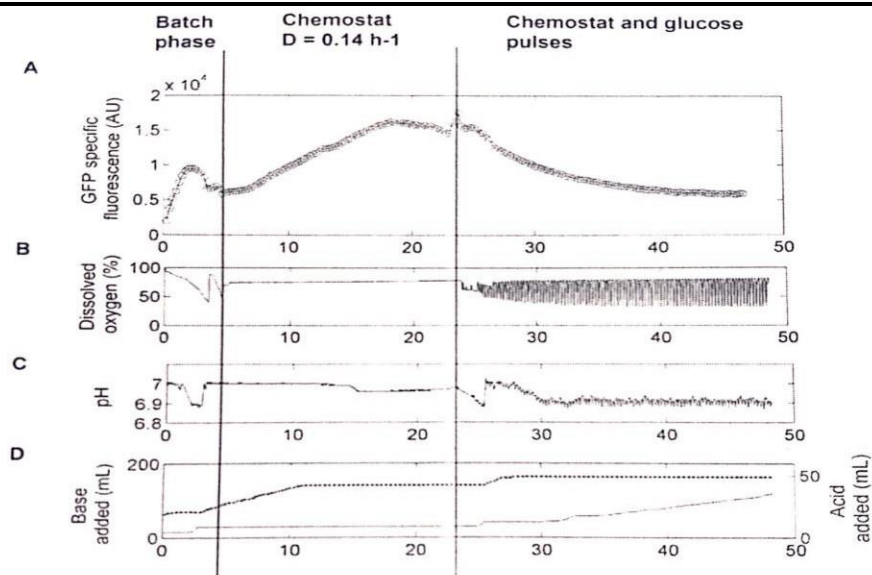
Figure' 3. Direct comparisons of on-line variables with GFP evolution (A) acquired by automated FC. Dissolved oxygen profile is shown in order to highlight the three distinct phases: batch, chemostat mode at U = 0.14 h' and chemostat mode D = 0.14 h with glucose oscillations (B) as well as p1-I profile (C) and addition of acid (dotted line) and base (solid line) profiles (D).

Representative result of three independent ultivations was shown.

After an initial batch phase, a chemostat mode was initiated at a dilution rate (D) of 0.14 h' and glucose pulses was performed at a given frequency to the stabilized chemostat culture. Further assessment of this sensitivity in the cheniostat experiment was thus conducted and the response f the GFP biosensor was followed by automated FC (Figure 3A). The oscillated condition can be visualized in the evolution profile of dissolved oxygen (Figure 3B and pH (Figure 3C). which fluctuated corresponding to the glucose pulses period.

During the batch phase, GFP synthesis is scen to be correlated directly to growth rate, the correspondence of which is known as he t squared' rule. This observation is consistent with off- line GFP analysis an can be efficiently validated with a higher number of sampling points with the automated FC. When shifted to the chemostat mode, GFP level is stabilized for a very short period of time (around 2 hours) and then the signal increased and continued throughout the stabilizing. This phenomenon is unexpected since GFP would keep constant level if the activation of the fis promoter were proportional to the growth rate. In our previous studies, we have shown that proteins are secreted into the extracellular medium when microbial cells are exposed to substrate limitation. This phenomenon is reported by several authors and is generally termed as protein leakage. Since the JIs promoter is up- regulated when cells are shifted from minimal to rich medium conditions, this release of proteins could possibly contributes to the increases of the pool of available amino acids (AA). This trend can be visualized on the basis of the pH regulation profile (Figure 3D), implying mainly the addition of acids in the first phases of the culture (from 3 to 12 hours). A significant advantage of the automated FC is that parameters related to cell physiology (such as GFP synthesis) could match on-line process variables in real time. In our case, this phenomenon is related to a slight increase of the pH above set point and a continuous activation of the acid pump, which can be the basis of designing new control loops for bioprocess monitoring. By using automated FC, the on-line parameters

can be directly correlated to the up- regulation of the Jis promoter and the accumulation of GFP. However, this tendency was reversed after approximately 1 5 hours of culuvation where the acid addition stopped, the pH dropped slightly and the accumulation speed of GFP signal ceased. This tendency is later confirmed over the course of glucose pulsing and the GFP signal even dropped back to the level lowcr than its starting point in the continuous culturing. Cell growth during this phase is n based on glucose consumption with a global acidification of the medium and thc activation of the base pump.

Substrate limitation induces a significant segregation of the popu'ation according to Pt uptake

The automated FC :stem used in this work allows for the determination of two physio1ogicall relat..d parameters. i.e. the intracellular GFP recorded in the FL! channel and the P1 up:ake intensity recorded in the FL3 channel. One of the advantage of FC is the possibJity to quantify single-cell parameters and thus to determine whether the distribm ion of this parameter is segregated or not in the microbial population. This segEegation is critical since it becomes clear now that for several recombinant systems, the productivity is generally related to a fraction of the population but not to the whole population. The availability of single-cell analytical tool is thus of importance at the process control level. In our previous operating conditions (Figure 3), no segregation was observed at the level of GFP synthesis. GFP is generally considered as a poor molecular tracer of physiological activity considering its slow degradation rate and even the destabilized version of the GFP used in our study cannot explain the appearance of segregation that not recorded in the FL 1 channel, due to the fact that a significant segregation of the population can be seen at the level of the P1 uptake intensity (Figure 4).
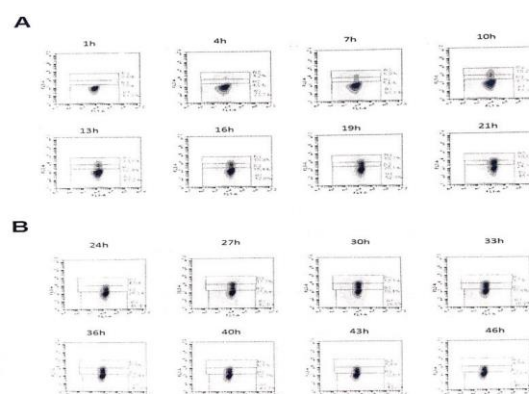
Figure 4. Flow cytometric dot plot for the evolution of subpopulations according to the FLI (GFP synthesis) and FL3 (P1 uptake) parameters in the batch and the chemostat phase (A) and the chemostat phase with additional glucose pulses (B).

The appearance of the subpopulations can be clearly visualized in the FL3 channel and is displayed as the subpopulation distribution.

When the cultivation shifted from the batch to the chemostat mode, the fractioning of microbial population appeared and can be clearly visualized in P1 profile in Figure 4A, resulting a progressive drop in the events of Ri region from 81% to 45.9% of gated E. coli cells in the first 21 hours' cultivation. The appearance of a R3 subpopulation exhibiting a higher red fluorescence that attributed to the uptake of P1 corresponds to a fraction of cells with damaged membrane. It is interesting to see that microbial cells are capable of adapting themselves to the limiting conditions. The observation of an "intermediate" subpopulation (R2) appeared and the R3 subpopulation progressively disappeared along the cultivation.

Clearly, two subpopulations were present in the glucose oscillation condition (figure 4B). This observation is important since the R2 subpopulation could be responsible for the protein leakage effect noticed at the level of the GFP synthesis. The intensity of segregation could not be quantified by looking at conventional flow cytogram that displayecf in figure 4, the quantification of this segregation will be addressed in the next section.

Segregation intensity can be quantified by using the mean to median ratio

In a process control perspective and considering its biological implications, it is important to pararneterize the segregation level of a microbial population by automated FC. The mean to median ratio (can be used to quantify the segregation since the median value is less affected by extreme values than the mean value in a population with the normal or non-normal distribution. As so, for our results of the non-segregated parameter, GFP. similar trends of inducement and decrease was shown for the median and mean values, whereas for the segre ararneter, P1. median value and mean value tended to be very different with rne P1 intensity lower than its medium, suggesting a dramatic variation in the population structure (Figure 5). With some discrepancy, the mean and the median value of P1 nsity kept rather constant after 10 hours culturing with a disturbance from glucose oscillation, which contributed to the observation of downshifts in both parameters.
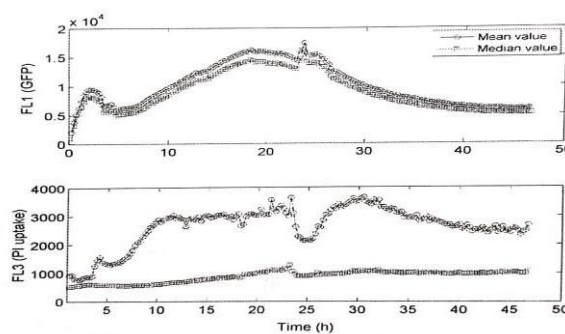

Figure 5. Time profile of the mean and the median value for both GFP and P1 intensity. Similar evolution profile of both mean and median is shown for the GFP fluorescence, but not for the P1 measurement. A disturbance is seen in the P1 intensity plot, corresponding to the glucose pulsing.
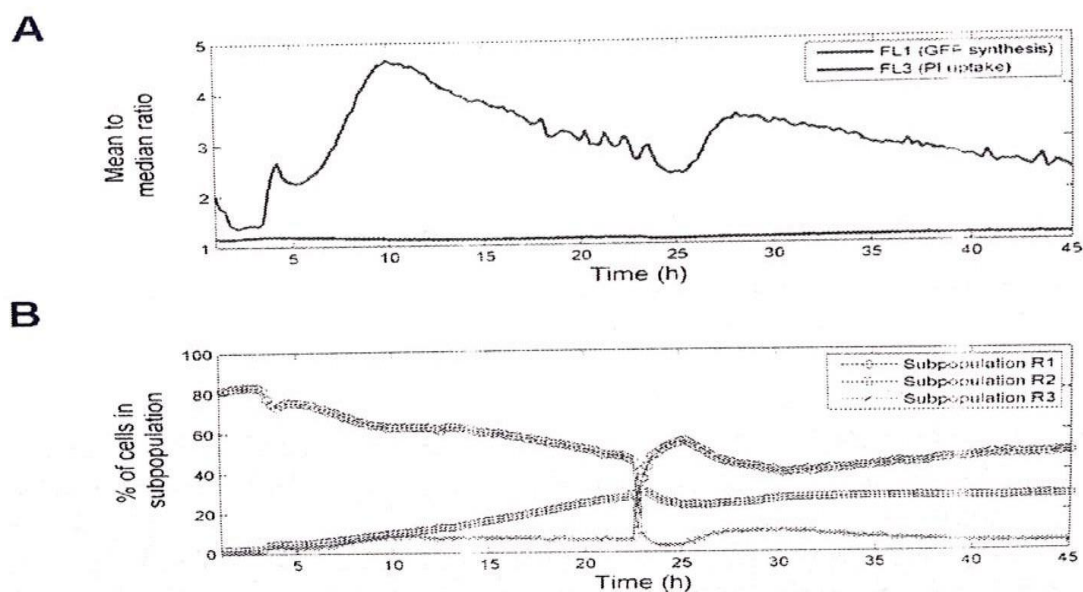
Figure 6. The mean to median ratio (MMR) of the GFP signal and P1 uptake intensity is shown over time (A), corresponding with the fractioning of population (B) distribution in three regions, namely, Ri (healthy cells), R2 (intermediate population) and R3 (damaged cells).

In figure 6, the initial phase of the segregation involves thv distribution of the cells between a Ri and a R3 subpopulation, corresponding respectively to healthy and membrane-compromised cells. As the cells approached the continuous cultivation phase, a significant increase of the MMR is seen, suggesting the elevated separation of Ri and R3. After this initial increment, however, cells adapt themselves to limitation and an intermediate R2 subpopulation appeared, which minored as the decrease of MMR with about 4.9% of cells falling into the R3 region, 3 3.6% in R2 region and 45.1% in Ri region. Glucose pulsing seemed contribute negatively to this adaption process supported by the jump of MMR (Figure 6A) and a larger population in R3 region (Figure 6B). It appears that the intensity of the segregation can be quantified by introducing the MMR parameter and on the other hand, the loss of GFP after pulsing may ascribe to the appearance of subpopulations.

Conclusion

The interest of the automated FC is to gather on-line physiologically related parameters and to allow direct comparison of these parameters with process variables. The algorithm integrating the residence time of microbial cells inside the sampling loop and the incubation time with the fluorescent stain can be easily modified in order to meet the requirement of other types of stains. The mean to median ratio tends to be another parameter to quantify population segregation. In conclusion, our low-cost automated flow-cytometry protocol is very efficient tool for tracking dynamic cellular response in the cultivation process and it also provides great potential of designing new feedback control system based on such as cell density, base or acid addition.

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<div align="center">**5.3 Manuscript 3**</div>

Shifts of population heterogeneity distributions in response to steady-state growth rate and glucose perturbations in Escherichia co/i and Saccharomyces cerevisiae

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Abstract

Two growth rate reporters of S. cerevisiae and E. co/i based on Green Fluorescent Protein (GFP). was characterized and used to investigate the effects of glucose pulses at single-cell level. A direct positive correlation between growth rate and GFP fluorescence could not be seen for either growth reporter. The E. co/i cells and slow growing yeast reacted to the glucose pulses with slight up-shifts in fluorescence, indicating that the gene regulations of the promoters primarily responded to environmental shifts. The effect of freezing on cell before sample analysis was also investigated. The yeast cells were seen to be heavily influenced by the freezing procedure at high dilution rates, whereas they were more resilient at low dilution rates. In addition, the glucose pulses had an instant effect in protecting parts of the cell population of the rapidly growing cells (D0.3 h') against freeze-thaw damage. This was not seen for the slower growing cells (0.05 h Novel methods of quantifying population heterogeneity were also developed. Using peak width and coefficient of variation (CV) of the population distributions, information on the spread of the population was obtained in a quantitative manner. The slope of cumulative distribution functions (CDF) plots was used to quantify shifts over time and differences in peak shape. Together these methods offered a less arbitrary way of quantifying flow cytornetry data and population heterogeneity. Using these methods it was seen that both faster growing yeast and E. co/i had a significant shift in heterogeneity in response to the glucose pulse, whereas the heterogeneity of the slower growing cells remained largely unaffected.

Keywords: Population heterogeneity, glucose pulse, reporter strain, freezing effect, flow cytometry

Introduction

Optimization of industrial cultivation processes requires a comprehensive analysis and understanding of the physiology of the host strain throughout the cultivation. Traditionally, this is performed on averaged cell characteristics from samples containing millions of cells.' However, microbial cells exhibit an intrinsic cell-to- cell variability that is both influenced by and affect the cultivation process. In addition, large scale cultivations introduce spatial heterogeneity through gradients of e.g. dissolved oxygen, substrate, temperature and pH exposing the microbial cells to rapid changes in environmental conditions as they circulate throughout the reactor. These dynamic conditions pose a stress on the cells, which affects their metabolism and the inherent population heterogeneity. Although this heterogeneity has been found to be harmful for cultivation processes by reducing yields and increasing byproduct formation, there are also indications that it may be beneficial as it facilitates a quick adaption to conditions." Transient metabolic responses of microorganisms to similar rapid changes in nutrient availability have been addressed in several studies. It has been shown that external perturbations can disturb optimal enzyme activity levels, disrupt metabolic fluxes, destabilize cellular structures, affect chemical gradients, influence the intracellular pH etc., leading to overall instability. The concomitant stress responses are transient at gene expression level, leading to new steady state levels similar to the unstressed cells even in the presence of a persistent stress.' The magnitude and duration of such transcriptional responses corresponds with the duration and magnitude of the perturbation.' Although stress responses have been studied, little is known about how changing environments effects population heterogeneity or how different sub-populations contribute to the cultivation process.

The aim of the present study is to gain deeper insight into how microbial population heterogeneity. is influenced by growth rate and changing nutrient availability. To enable this. reporter strains for the industrially relevant model organisms Saccharornvces cerevisiae and Escherichia co/i were employed and the cell responses vere analyzed at single cell resolution. The reporter systems are based on expression of green fluorescent protein (GFP) coupled with ribosomal or ribosomal related amoters. The state of the individual cells is then reflected in the expression of the reporter protein, which can be followed by its fluorescence by using flow cytometry. In addition, information about changes in cell size is obtained from the flow cytometer forward scatter (FSC) signal, which normally correlates with particle size.

The integrated S. cerevisiae reporter system (FE440) is based on expression of the stable eGFP under the control of the ribosomal RPL22a promoter. This promoter was chosen as its expression has been shown to be growth correlated in a transcriptome analysis study.' In addition, the reporter system allows the analysis of cell membrane robustness as a loss of GFP signal in cells has been seen to be coupled with permeabilised cell membranes. These two abilities make the reporter strain an interesting tool for further investigations of how cell robustness and growth rate population distributions respond to changes in nutrient availability.

The E. co/i reporter system is of a slightly different nature expressing an unstable fluorescent protein from a low copy number plasmid. The promoter reported on, the so-called JIs promoter. controls the expression of a transcriptional activator that in turn activates the bacteria ribosornal promoter rrnB P1. The reporter strain MG1655/pGS2OfisGFPAAV was selected based on its ability to rapidly respond to changing environmental conditions and its high fluorescence signal as seen in a prior trial in a heterogeneous reactor. It has earlier been demonstrated that the behaviour of the reporter system depends on the operating mode of the

bioreactor and that the reporter gives different responses when exposed to either short-term or long-term nutrient limitation and may hence be used to evaluate cellular behaviour in heterogeneous bioreactors, where cells experience dynamic environmental conditions.

Using the described reporter systems in continuous cultivations growth rate related effects on population heterogeneity were investigated. Glucose perturbations mimicking the conditions cells experience in industrial scale bioreactors were introduced to steady-state cultivations operating at different dilution rates and the concomitant cellular responses studied. The aim was to increase the understanding of microbial behavior at the single-cell level, which might provide important insight for the generation of novel strategies to improve the performance and robustness of industrial scale cultivations.

Materials and Methods

S. cerevisiae reporter strain FE440 and F. co/i MGI 655/pGS2OPfisGFPAA V was used throughout this study. in F. co/i cultivation, 25 j.ig/mL of chioramphenicol (stock concentration: 50 mg/mi in 96% ethanol) was added to medium to keep the selection pressure.

Cultivation conditions

Pre-culture. Saccharomyces cerevisiae: A single colony of the growth reporter strain FE440 was picked from a plate with minimal medium and used to inoculate a 0.5 L baffled shake flask with 100 ml of defined mineral medium containing 7.5 g/L (NH 14.4 g/L KH 0.5 g/L MgSO 2 ml/L trace metal solution, 1 ml/L vitamin solution and 10 gIL glucose. The pre-culture was incubated in an orbital shaker set to 150 rpm at 30°C until mid-exponential phase (approximately 10 h) and directly used for inoculation.

Escherichia co/i: The F. co/i inoculum was obtained by a two-step procedure. First. a single colony of MG1655/pGS2OPfisGFPAAV from a fresh LB plate was used to inoculate a 100 mL Luria Broth containing shake flask at 37°C and grown overnight A dilution series with 10-fold steps was prepared from the overnight culture and incubated at 37°C for 6 to 8 hours on an orbital shaker (180 rpm). The optical density from the dilution series was subsequently measured and the one with an 0D between ft4 and 0.6 was used to inoculate the bioreactors.

Chemostats. S. cerevisiae: Aerobic level-based chernostats with S different dilution rates (D= 0.05/0.1/0.2/0.25/0.3 h were performed in duplicates us.ng 1L bioreactors (Sartorius. B. Braun Biotech International, GmbH. Melsungen. Germany) with a working volume of IL. pH and DOT electrodes (Toledo. 01-1. USA) were calibrated according to standard procedures provided by the manufacturer using two point calibration (pH 4 and 7. gassing with oxygen (100%) and nitrogen (0%). respectively). The growth medium was a defined mineral medium according to Verduyn ci a/. (1992)21 with 5 g/L glucose. The 0D for inoculation was 0.001. The pH was adjusted and kept constant at 5.0 using 2M aOH. Temperature, aeration and stirring were kept constant at 30°C, 1 v/v/rn and 600 rpm.

- E. coil: chemostat cultures were inoculated with an 0D of 0.01 and operated at 37

°C in the same bioreactor setup but with a working volume of 0.6 liter. The growth medium was a defined mineral medium containing 4.5 gIL glucose, 2 g/L Na 2.468 g/L (NH 0.5 g/L NH 14.6 g/L K 3.6 g/L NaH 1 gIL (NH 50 iL Sigma 204 Antifoam and 0.1 g/L thiamine. After autoclaving, 3 m MgSO 1 rnL trace metal solution was sterile filtered (0.2 im) into the medium. The trace metal solution contained 0.5 gIL CaCI

0.18 g/L ZnSO 0.1 gIL MnSO 20.1 gIL Na 16.7 gIL FeC1 0.16 g/L CuSO and 0.18 gIL C0C1 Aeration and agitation speed were kept constant at 1 v/v/rn and 1000 rpm to avoid oxygen limitation.

The batch phase of both yeast and E. coil culture was followed by 0D measurement and continuous analysis of the off-gas composition by a Mass spectrometer (Prima Pro Process MS, Thermo Fisher Scientific, Winsford UK). Steady state was considered established when dry weight, dissolved oxygen tension (DOT), HPLC metabolites and exhaust gas concentration (C0 had remained constant for at least three residence times. After glucose depletion, detected as a rapid drop in the CO content of the off gas, the cultures were switched to chernostat by applying a feed with the same medium as used for the batch with the desired dilution rate. The volume was kept constant by a level based outlet. After confirming steady state all cultures were perturbed with a concentrated glucose solution corresponding to a final bioreactor concentration of I g L for S. cerevisiae and 0.45 g/L for E. co/i using a sterile syringe and followed by frequent sampling (time depending on the organism, see result section).

Samples were withdrawn for 0D high performance liquid chromatography (HPLC). dry weight (DW) and flow cytometrv analysis. Samples for 0D and DW were analyzed directly, HPLC samples were sterile filtered and stored at -20 °C. Samples for flow cytometry were mixed with 1ycerol to a final concentration of l5°/ and frozen and stored in a -80 °C freezer. For east, flow cytometry analysis was also performed on fresh samples for two of the dilution rates (D= 0.05 h and 0.3 h'), where broth was mixed with 15 glycerol ani kept on ice until analysed (maximum an hour).

Freezing test of E. co/i samples

To investigate the physical effect of different sample treatments on GFP detection, a simple freezing test was perfoued in shake flask using same minimum medium as in chemostat cultivation. Triplicate samples were collected from each growth phase and each sample was divided into three sub-samples, one was measured directly, one instantaneously frozen in liquid nitrogen with 15% glycerol and one more slowly frozen in a -80°C freezer, also with 15% glycerol. To avoid non-desirable degradation during the sample treatment, 10 samples were thawed at a time, then centrifuged, and re-suspended in 0.9% NaCI. The maximum lag time between the first and the 10th analyzed sample was 10 mm, during which we assume that the intracellular GFP concentration did not influenced by the sample preparation.

Sample analysis

OD, DW and HPLC. Growth was monitored by measuring 0D with a Shimadzu UV mini 1240 spectrophotometer (Shimidzu. Kyoto, Japan). Dry weight measurements were performed on 5 ml cultivation broth according to Olsson and Nielsen (1997)23 The concentration of glucose, acetate, ethanol, glycerol and pyruvate in the broth of the Saccharonivces cerevisiae cultivation was determined by HPLC as earlier described by Carlquist et a/. (201 2).6 The E. co/i cultivations were in addition analyzed for lactate and fonnate, and the glucose concentration was measured by enzymatic calorimetric procedure (ABX Pentra Glucose HK CP).

Flow cytotnetrv. A FA(SAria' III (Becton-Dickinson. NJ. USA) flow cytometer was used for single-cell analysis of both yeast and bacteria. Excitation wavelength for the laser was set to 48 nm. Two scattering channels (FSC and SSC) and two fluoresceni celection ch were used in the analysis. Fluorescence emission levels were measured usng a band pass filter at 530±30 nm and 610 nm. Light scattering and fluorescence

levels were standardized using 2.5 irn fluorescent polystyrene beads. Sampies for flow cytometry were centrifuged for 1 mm at 3000 g and 4 °C, resuspended in 0.9 ° saline solution and directly analysed. 20,000 and 30,000 events were recorded for yeast and E. co/i respectively. CS&T beads (Cytometer Setup and Tracking beads) (Becton Dickinson, USA) were used for the automated QA QC of the machine performance.

Data analysis. Processing and analysis of the flow cytornetry raw data was performed using MatLab ® R2010b (The MathWorks, Inc., Natick, MA, USA). The raw data was extracted as fcs files and loaded in MatLab with the help of the readfsc function (by L. Balkay, University of Debrecen, Hungary, available on matlab central file sharing). The HPLC data was collected in an excel file and imported into MatLab. The data from the fcs files was extracted and saved into mat files including the recorded GFP fluorescence and the FSC for each steady state and pulse experiment. The cumulative cell distribution from the 1024 recording channels of the flow cytometer was fitted to the data of the GFP fluorescence using the hist function and afterwards applying the cdfplot function. By applying the hist function to the 1024 recording channels cell count was saved for all channels and plotted as channel number fluorescence for the GFP detector. Biplots for FSC and GFP were created using this function. For better quantitative data for the distributions of GFP, the peakwidth at baseline level was calculated by searching for the borders of the peak, which were considered as 25 cell counts per channel number to disregard the noise level of the flow cytorneter. By substracting the channel numbers for the higher and lower bOrder the peakwidth as determined. Furthermore, the mean function was used to calculate the Mean FSC and Mean GFP fluorescence. The normalized GFP was estimated by dividing the mean GFP by the mean FSC. By dividing the peak'.vidth by the Mean GFP the coefficient of variance of the distribution was generated. Finally the slope of the cdfplot was estimated by fitting a line to the exponential part of the cumulative distribution of the GFP fluorescence histograms by using the polyfit function with a degree of one. For frozen pulse samples the sub- population percentage was computed by dividing the histogram plot into three areas, a low middle and a high fluorescent. The areas were set in relation to the local minumim in the histogram between two sub-populations, which was calculating using the miii function. The middle fluorescence range was excluded to avoid biased data and then the sub-population percentage in the low and high range was calculated by diviJing the cell amount in the sub-population by the total cell amount found in the two fluorescence ranges. Due to having duplicate datasets for all samples, all values and stirnated parameters were stated showing replicates.

Table 1. List of abbreviations

Symbol	Description	Unit
cdf	Cumulative distribution function	-
CV	Coeffcient of variabtion	[%]
D	Dilution rate	h^{-1}
FSC	Forward Scatter	-
GFP	Green flourescent protein	-
rs	Specific substrate uptake rate	[g glucose/gcells/h]
rerthanol	Specific ethanol production rate	[g ethanol/g cells/h]
RT	Residence time	(h)

SSC	Side Scatter	-
Ysx	Growth yield on the feeded substrate	[cmole/cmole]
Ysco2	Yield of CO2 on the fedded substrate	[cmole/cmole]
YSEth	Yield of ethanol on the feeded substrate	[cmole/cmole]
YSGly	Yield of glycerol on the feeded substrate	[cmole/cmole]
YSAce	Yield of acetate on the feeded substrate	[cmole/cmole]
Yspyr	Yield of pyruvate on the feeded substrate	[cmole/cmole]

Results

To investigate how microbial population heterogeneity is influenced by growth rate and glucose excess, glucose-limited continuous cultivations with glucose pulses where performed using reporter strains of S. cercvisiae reporter strain FE440 and E. co/i MG165 Based on that we wanted to compare the population response for cells in fully respiratory versus respiro- fermentative growth, continuous cultivations at low and high dilution rates were performed for both yeast and E. co/i. The low dilution rates used correspond to approximately 13% of the maximum specific growth rate, i.e. 0.05 and 0.09 h for S. cerevisiae and E. co/i respectively, and the high dilution rates correspond to approximately 77% of the maximum specific growth rate, being 0.3 and 0.53 h' for S. cerevisiae and E. co/i respectively. In addition, for E. coli one additional dilution rate, 0.44 h just around the rate where overflow metabolism is believed to set in, was also included and for yeast three dilution rates in between those chosen as low and high were included in the evaluation of influence of growth rate on level of heterogeneity.

Physiology of cultivations for S. cerevisiae and E. coli

Yields on glucose in steady state yeast cultivation were calculated for cell mass, C0 ethanol and acetate as well as the uptake and production rate of glucose and ethanol was shown in table 2. In Table 3, yields on glucose in steady state for E. co/i cultivation for cell mass, CO and all metabolites produced in significant amounts, as well as the uptake rate of glucose were calculated. Carbon balances were calculated from the yield coefficients to test data consistency.

Table 2. Yields (ratios of cell mass, C0 ethanol and acetate produced on amount of consumed substrate) and carbon balance including standard deviation, as well as consumption respectively production rates for glucose and ethanol for aerobic glucose- limited continuous cultivation of S. cerevisiae FE440 performed with different dilution rates (D= O.0510.1IO.210.2510.3 h5. All data are presented as mean values including standard deviations and originate from two independent cultivations and, for each cultivation samples were taken over a minimum of three different residence times.

Chemostat parameter	D=0.05h-1	D=0.1h-1	D=0.2h-1	D=0.25h-1	D=0.3h-1
YSX [cmole/cmole]	0.58+/-0.02	0.56+/-0.06	0.60+/-0.01	0.35+/-0.02	0.22+/-0.01
YSC02[cmole/cmole]	0.53+/-0.02	0.40+/-0.4	0.38+/-0.01	0.35+/-0.01	0.27+/-0.01
YSEth[cmole/cmole]	-	-	-	0.22+/-0.01	0.42+/-0.01
YSAce [cmole/cmole]	-	-	-	-	0.01+/-0.00
rs[g/gh]	0.10+/-0.01	0.20+/-0.02	0.38+/-0.00	0.28+/-0.05	1.61+/-0.05

rEthanol [g/gh]	-	-	-	0.16+/-0.00	0.52+/-0.00
Carbon balance	**1.11+/-0.05**	**0.96+/-0.10**	**0.98+/-0.01**	**0.94+/-0.03**	**0.92+/-0.01**

For yeast, the dilution rates below D= 0.25 h all showed similar yield on biomass, Ysx, around 55-60%, which is consistent with literature data for respiratory metabolism. Also, no accumulation of metabolites was detected, indicating an absence of overflow metabolism, which is in accordance with earlier observations. For the two higher dilution rates (D= 0.25 h and 0.3 h'), Ysx was significantly lower and was accompanied by accumulation of ethanol as well as small amounts of acetate for the highest dilution rate (D= 0.3 h'). This is in agreement with results found in another study where a critical dilution rate for onset of respiro-fermentative metabolism has been found to be 0.28 h in CEN.PK113-7D. The YSCO2 decreased with increasing dilution rate, whereas rs as well as rEthanol increased, which also are in agreement with Postma et al. (II 989).27 In general; all yields showed low standard deviation and thereby confirmed steady state on the physiological level and high reproducibility between replicates. Carbon balances for all the cultivations closed with a maximum error of 11%.

For E. co/i, biornass yields on glucose were about 0.6 cmole/cmole for the low dilution rate and 0.65 cmole/cmole for the higher ones. These results correspond well with literature data on E. co/i K12 where an Ysx of about 0.6 cmole/cmole for lower dilution rates, which then increased with increasing dilution rate to reach a maximum of 0.69 cmole/cmole before tapering off at even higher rates. Since ethanol was present in the medium (added as a solvent for chloramphenicol) negative yields on ethanol was obtained when it was consumed. Similarly to S. cerevisiae, at the low growth rate (D=0.09 h E. co/i cultivations a strictly respiratory metabolism with no acetate formation was observed. Literature data suggests a shift to respire fermentative growth at a dilution rate above about 0.4 h' with concomitant acetate production. However, this was not seen in the present study as only small amounts of acetate were observed for the higher growth rates, instead pvruvate accumulated. This observation is consistent with Qiang Hua ci a! (2004) where no acetate excretion was found at detectable levels, conversely their biomass yields were found to be lower and CO yields higher compared to our data. Lactate and formate vere only seen in very low concentrations in the medium (data not shown). The low standard deviations for the calculated yields, in addition to that carbon balances closed with a maximum error of 5%. demonstrate high data consistency for the present study.

Table 3. Yield coefficients and carbon balance as well as glucose consumption rate at different dilution rates for aerobic glucose-limited chemostats of E. co/i MG1655/pGS2OPfisGFPAAV (+/- standard deviation). Data are presented as mean values and originate from samples taken over at least three different residence times. Each dilution rate was carried out as a biological duplicate (D=O.09 h or as a single measurement (D=O.44 h' and D=O.53 h').

Chemostat parameter	D=0.09h-1	D=0.44h-1	D=0.53h-1
Y_{SX} [cmole/cmole]	0.59+/-0.02	0.65+/-0.01	0.65+/-0.01
Y_{SCO2} [cmole/cmole]	038+/-0.009	0.31+/-0.002	0.30+/-0.003
Y_{SEth} [cmole/cmole]	-0.01+/-0.009	-0.3+/-0.02	-0.03+/-0.02
Y_{SAce} [cmole/cmole]	-	0.02+/-0.04	0.017+/-0.004
Y_{SFor} [cmole/cmole]	0.020+/-0.002	0.029+/-0.001	0.022+/-0.0005
Y_{spyr} [cmole/cmole]	0.18+/-0.09	0063+/-0.04	0.083+/-0.009
rS[g/gh]	0.18+/-0.09	0.78+/-0.04	0.94+/-0.00
Carbon balance	**0.98+/-0.02**	**1.05+/-0.03**	**1.04+/-0.02**

For all cultivations a pulse of glucose was added after steady state had been reached. in order to investigate population heterogeneity in steady state and after glucose perturbation to S. cerevisiae and E. co/i cultures, single cell analysis using flow cytometry was applied. The distribution of individual cell responses is presented in the following sections.

GFP fluorescence distribution for S. cerevisiae cells at steath -state conditions

GFP fluorescence versus forward scatter (FSC) distributions (figure 1 A and B) for the two dilution rates. D= 0.05 h and D= 0.3 h* showed resemblance, but a slightly different subpopulation pattern was seen. For both dilution rates population fluorescence varied within an interval between 100-600 fluorescence channel numbers and the cells grouped into two subpopulations, one with high fluorescence and one with low. However, for the low dilution rate cultivation, the high fluorescence subpopulation was covering a broader fluorescence range (approx. 300- 600 fluorescence channel numbers) compared to the high dilution rate cultivation (approx. 400-600 fluorescence channel numbers). The FSC. which usually correlates with particle size. was seen to be within a range of 800-1000 for the majority of cells at both dilution rates. However, at D=0.05 h a minor subpopulation with smaller cell size, FSC=300-600, was observed.

Additionally to the above discussed characteristics of the GFP expression and distribution, quantitative and more descriptive parameters can be generated to facilitate comparison of flow cytometry data obtained for cells from different conditions. We have chosen to introduce coefficient of variation (CV) for mean GFP, peakwidth and the slope of the cumulative distribution curve as parameters describing population heterogeneity (table 4).

In general the fluorescence distribution for cells grown at a high dilution rate is more narrow and distinct, compared to the distribution for cells grown at a low dilution rate (figure 1E and F), which can also be confirmed by the peakwidth (table 4). Additionally. more noise in the distribution was seen for the low dilution rate. This indicates a higher degree of population heterogeneity for growth at low rates. Another way to look at the cell fluorescence distribution is to plot the cumulative distribution and by calculating its slope, which can be used to quantify heterogeneity (cdf-plot, figure IG and H and table 4). The lower slope seen for D=0.05 h' (0.0072 2.24 10- compared to 0.0101±- 5.20i0 for 0.3 h') indicates a broader distribution for the

low dilution rate. Mean GFP fluorescence was rather similar for the two dilution rates, 456.9+!- 28.3 and 464.6+- 20.3 fluorescence channel numbers. for D=0.05 and 0.3 h respectively. however, the slightly higher CV of the GFP distribution for the low dilution rate further confirms a higher level of heterogeneity at D=0.05 h After normalizing mean GFP fluorescence with mean cell size. i.e. fluorescence per cell size unit, the value obtained for D= 0.05 h' (0.49) and D= 0.3 Ii (0.50), is almost the same. Which also proves to be the origin of the almost same mean GFP fluorescence as they have almost identical mean cell sizes.
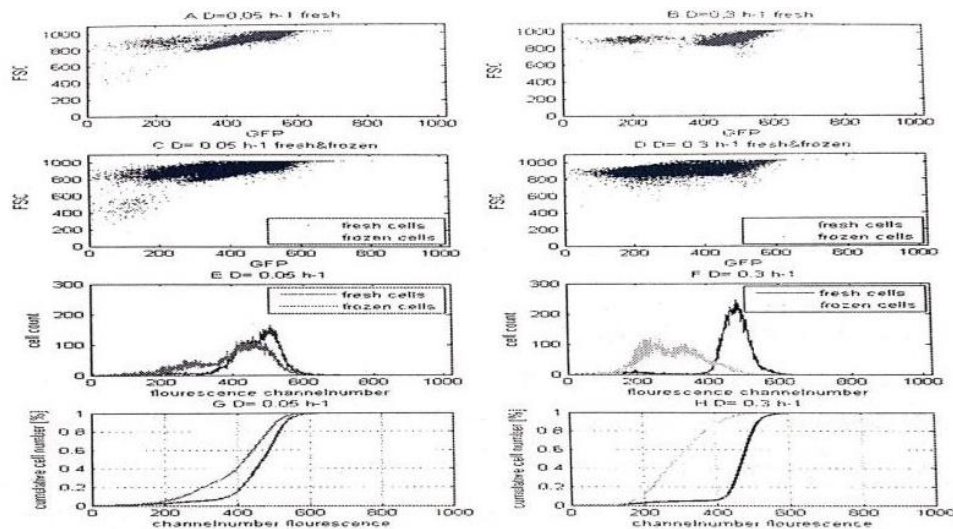

Figure 1. Fluorescence and size distributions for fresh and frozen yeast cells at steady-state. conditions: Biplots for FSC against GFP for fresh cells grown at D= 0.05 h (A) and D= 0.3 h (B) as well as for comparison between fresh and frozen cells for both dilution rates (C and D). Also GFP fluorescence represented as cell count against channel number fluorescence (E and F) and cdfplots of cumulative cell number against channel number fluorescence (G and H) for D= 0.05 h and D= 0.3 h for fresh and frozen S. cerevisiae FE440 cells in steady state in aerobic glucose- limited chemostat. The plots represent average values from samples taken after three different residence times.

Table 4. Heterogeneity at steady-state conditions for fresh yeast cells: quantitative values for measure of GFP fluorescence heterogeneity f D= 0.05 h and D= 0.3 h represented as Mean GFP with the belonging CV. thc slope of cd plots and peakwidth

Parameter	0.05	0.3
Mean GFP	456.9+/-28.3	464.6+/-20.3
CV GFP	0.18	0.16
Slope cdf plot	0.0062+/-2.2.10-4	0.0095+/-5.2.10-4
Peakwidth	271+/-15.6	227+/-42.4

Cell robustness to freeze-thaw stress for cells from steady state conditions

The S. cerevisiae reporter allows the identification of subpopulations with high and low cell membrane robustness to freeze-thaw stress as an inverse correlation of GFP signal and membrane integrity has been found. The underlying reason might be a reduction in fluorescence due to a shift towards lower intracellular pH due to leaking membranes and an extracellular pH around 5.5. We investigated this phenomenon by permeabilizing yeast cells with Amphotericin B and incubating in buffers with different pH

before analysis on the flow cytometer. A linear decrease in fluorescence was observed as the external pH dropped below 7 (data not shown). To investigate how growth rate affects membrane integrity, samples from the five dilution rates D=0.05/0.1/0.2/0.25/0.3 h were frozen in 15% glycerol at -80°C before flow cytometry analysis and data compared to data from fresh cells.

Cells growing at a high and low dilution rate, respectively, are differently affected by freezing, as can be seen from the FSC-GFP biplots and fluorescence histograms for fresh and frozen cells in figure IC to F. For D 0.05 h' the shape of the distribution as well as the fluorescence range remained the same after freezing and only a slight increase in the lower fluorescence population could be seen. However, for D= 0.3 h the fluorescence distribution got broader and the fluorescence was also considerably shifted towards lower values. As a result, the mean GFP fluorescence remained almost the same with freezing for D= 0.05 h' (409.0 - but decreased for the high dilution rate to 295.4 —/- 6.9 (see also figure 2, indicated by red circles). This leads to the conclusion that cells growing with a low growth rate (D 0.05 h are more robust towards freeze-thaw stress.To further investigate how growth rate influences freeze-thaw stress tolerance and the level of population heterogeneity: mean GFP. mean GFP nonnalized by cell size as well as CV of mean (iFP. slope of the cdf plot and peakwidth were calculated for all 5 dilution rates and plotted in figure 2. A similar mean GFP fluorescence (figure 2A) was found for cells from all dilution rates, except for D= 0.05 h where the mean fluorescence was higher. From [data it was seen that the cell size decreased with increase in growth rate until the dilution rate where respiro- fermentative metabolism sets in, and then sharply increased afterwards (data not shown). To remove the effect of cell size change on mean fluorescence. normalization with cell size was performed.

A steep increase in the normalized mean GFP was seen when going from 0.05 to 0.1 h Thereafter, the value remained almost constant for growth rates up to the dilution rate where respiro- fermentative metabolism sets in (0.25 h'). Interestingly, above this growth rate, the normalized GFP fluorescence decreased sharply.

The CV of the GFP distribution, giving indication of the level of heterogeneity for the population, showed a similar trend as for the normalized mean GFP, though in the opposite matter, suggesting a higher level of heterogeneity for D0.05 and D0.3 h than for the in between dilution rates. In general the GFP fluorescence distribution for cells grown at 0.1 h and 0.2 h' was more narrow (peakwidth, figure 2B). This can also be seen in the cdfplot which has the highest slope for D= 0.1 h and a bit lower value for 0.2 and 0.25 h' (figure 2B). For D= 0.05 h and 0.3 h' the GFP fluorescence distribution is broader, resulting in bigger peakwidth compared to the other dilution rates.

Though the peak characteristics are different for D= 0.05 h and 0.3 h the slope of the cdfplot is similar for these two growth rates (figure 2B). Compared to fresh cells (flgure 1E). the distribution for frozen cells grown at 0.05 h' was broader with a more distinct low fluorescent subpopulation, though keeping the same shape, resulting in a similar value of the slope of the cdfplot. For 0.3 h the slope of the cdfplot for frozen cells was considerably lower as the distribution shape changed with the big tailing towards higher fluorescence and a di\ision of the peak into two subpopulations (figure IF).
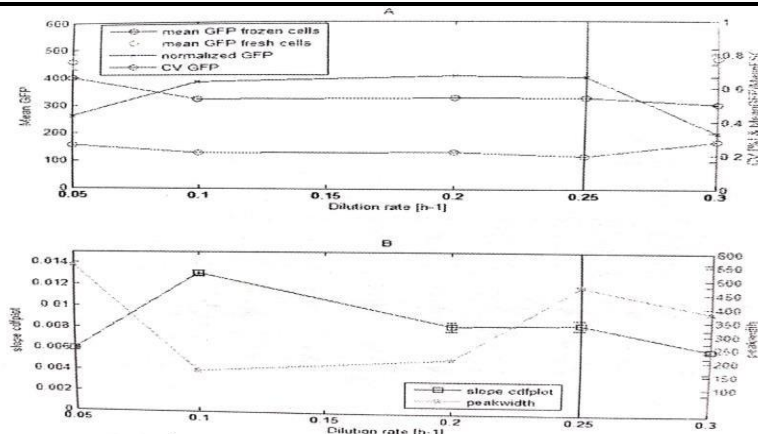
Figure 2. The effect of growth rate on fluorescence and heterogeneity for yeast cells exposed to freezing: Mean GFP fluorescence distribution as well as GFP fluorescence normalized by cell size together with the belonging coefficient of variance (A) as well as the peakwidth and the slope of the cdfplot (B) for S. cerevisiae cells at steady-state conditions in aerobic glucose-limited chemostat for D= 0.05 h', 0.1 h 0.2 h', 0.25 11' and D= 0.3 h'. The plots represent average values from samples taken after three different residence times. The blue line in both plots is indicating the dilution rate for which, as shown by the data, respiro-fermentative metabolism sets u-i.

Physiological response of S. cerevisiae to glucose perturbation

A glucose pulse oil g/L was introduced to the steady states at the different dilution tes and FSC. GYP fluorescence and HPLC data re-orded over the duration of each perturbation.
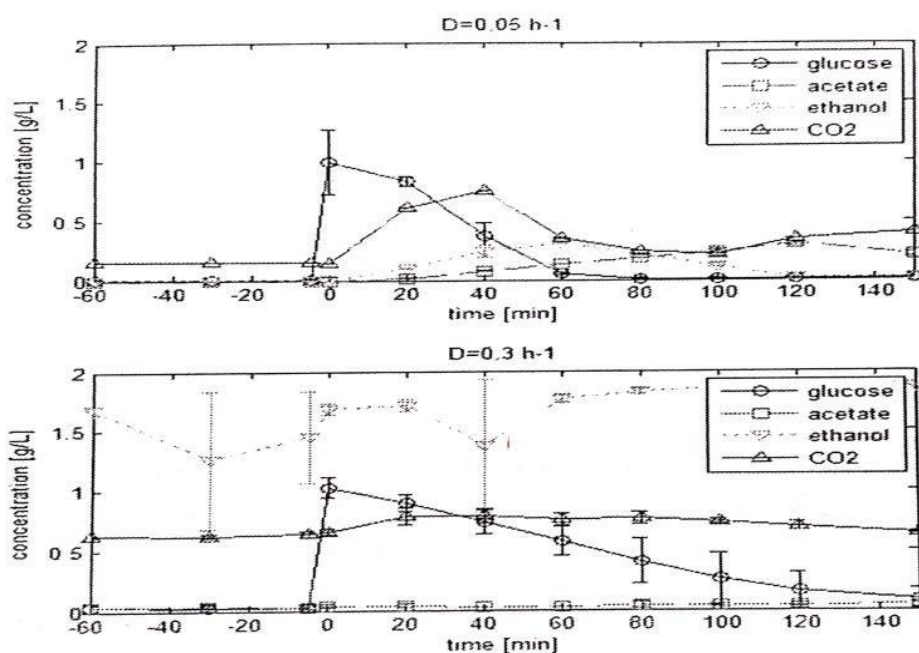
Figure 3. Time profiles of physiological response to a I gIL glucose pulse in aerobic glucose- limited chemostats of S. cerevisiae growing with a dilution rate of 0.05 h .(top) and 0.3 h' (bottom). Glucose (g/L). CO (v/v %), ethanol (g/L) and acetat & (g/L) concentrations are shown during the duration of the perturbation.

The lower dilution rates, 0.05 h' (figure 4) as well as 0.1 h and 0.2 h' (data not shown) displayed a batch like behavior with overflow metabolism, concomitant ethanol and acetate accumulation. as also earlier seen by Visser et al. (and a sharp increase in CO production when perturbed with a 1 g/L glucose pulse. In

contrast, the respiro-fermentative chemostats (0.25 and 0.3 h growing above the critical dilution rate, consumed the extra glucose at a much slower rate, did not react with an increased ethanol or acetate production and only showed a very slight increase in CO generation.

Influence of glucose perturbation on S. cerevisiae GFP fluorescence

Figure 4 shows the responses of the D= 0.05 h and 0.3 h' steady states to a 1 gI glucose pulse with regards to GFP fluorescence. From the fluorescence histogram ata for fresh cells (figure 4 top) it can be seen that the pulse caused a slight shift towards higher fluorescence for cells grown at 0.05 h whereas cells grown at 0.3 h here not influenced. This is also seen in the mean fluorescence, which remained constant for the higher rate and increased for cells grown at the lower rate afier around 30-40 mm after the pulse (figure 5A fresh). CV and peakwidth (figure SB and C fresh) indicate a somewhat higher heterogeneity for the low growth rate, however, no influence of the pulse was observed, as the values were constant over time. On the contrary, the slope of the cdfplot (figure 5D fresh) increased as a consequence of glucose perturbation for D=0.3 h' and decreased for D0.05 h'. The change in the slope of the cdf plot may be attributed to a decline in the low fluorescence subpopulation for cells grown at D0.3 h' and an increase for cells grown at D=0.05 h (see figure 4), whereas the main subpopulation remained constant. Coinciding with glucose depletion (compare with figure 3) the slope returned to the steady state value for both dilution rates.
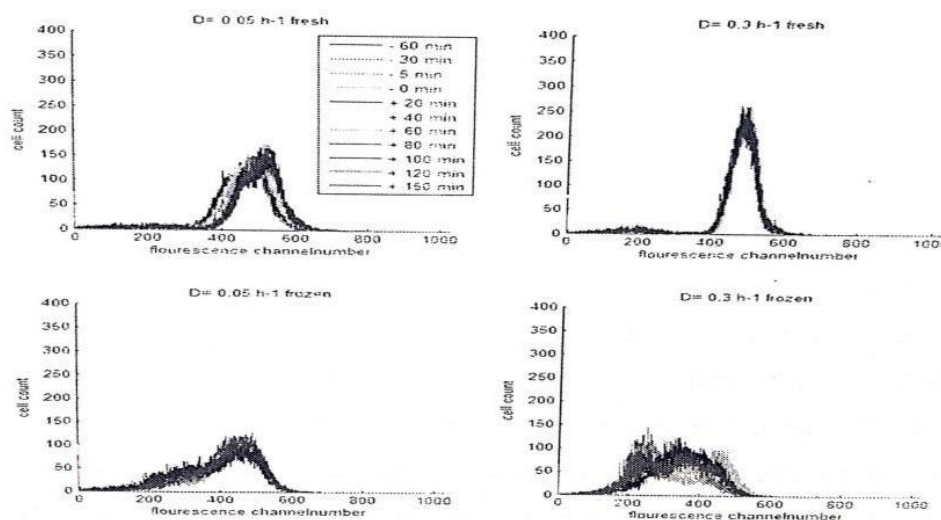
Figure 4. Time profiles of GFP fluorescence distribution for S. cerevisiac fresh cells and cells exposed to freezing: GFP fluorescence represented as cell count against channelnumber fluorescence of S. cerevisiae FE440 before. during and after a 1 g L glucose pulse in aerobic glucose-limited chemostats for D= 0.05 h (left) and D= 0.3 h (right) shown as comparison for fresh (top) and frozen cells (bottom). The plots each represent time points between -60 mm to +150 mm after the pulse.
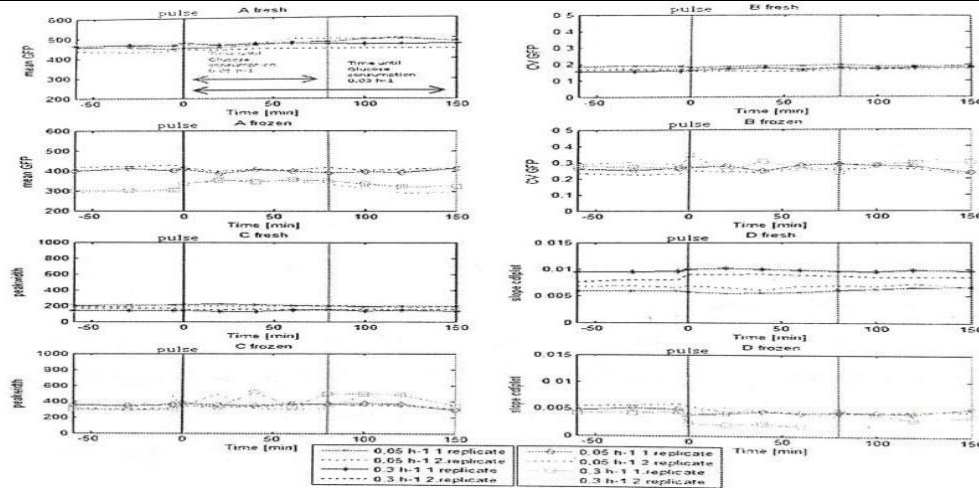
Figure 5. Heterogeneity dynamics following a glucose perturbation for fresh and frozen yeast cells: Mean GFP fluorescence (A) as well as CV of GFP fluorescence (B). peakwidth (C) and slope of the cdfplots (D) plotted against time following a I gIL glucose pulse for S. cerevisiae FE440 in aerobic glucose-limited chernostats for D= 0.05 ha and D= 0.3 ha for fresh (red and blue) and frozen cells (magenta and cyan). The red (respectively magenta) and blue (respectively cyan) colored vertical lines indicate the time when the pulsed glucose is consumed for 0.05 h respectively 0.3 ha, whereas the black line is indicating the time of the glucose pulse. Dotted lines show values for replicate cultivation.

Influence on cell robustness by glucose perturbation of S. cerevi cultivations

Comparing fluorescence histograms for fresh and frozen cells for D h and D=0.3 ha a distinct difference could be seen for the two dilution rates. For cells grown at the low dilution rate, GFP fluorescence distributions for cells exposed to a glucose pulse and frozen before analysis, u crc similar to those of cells from steady- state conditions. Hence, glueose perturbation did not influence the membrane integrity of cells grown at 0.05 ha (figure 4 and 5). On the contrary, the pulse significantly affected cells grown at 0.3 ha. The high fluorescence subpopulation increased as a response to the pulse (figure 4), which also was reflected in the increased mean GFP value (figure 5A frozen). Even though the mean GFP still was more reduced compared to fresh cells for D=0.3 h than for D=0.05 h addition of glucose seemed to some extent protect fast growing cells towards freeze-thaw stress.

To further elucidate the effect of the pulse on subpopulation distribution, the relative fraction of the low and high fluorescence sub-population was calculated at the different time points (figure 6). The low fluorescence sub-population contributed with 80% and the high fluorescence sub- population with 20% during steady-state conditions for cells grown at 0.3 h'. When glucose was added, this changed to an approximately equal distribution between the two sub-populations. For cells grown at 0.05 h the sub-population distribution was unaffected by the pulse and stayed at being approx. 20% of the low fluorescence population and approx. 80% of the high fluorescence subpopulation.
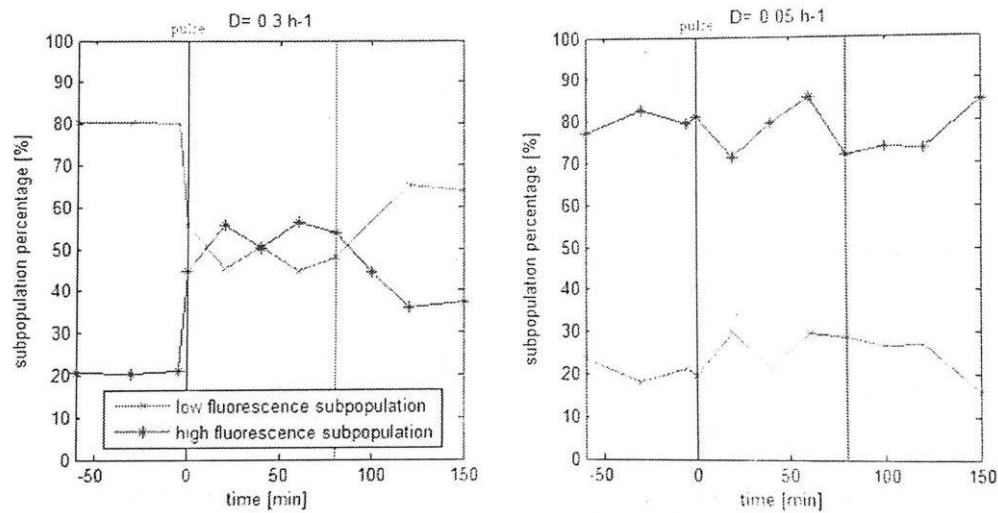
Figure 6. Subpopulation distribution (lvnamics following a glucose perturbation of yeast cells frozen before analysis: percentage for low (red) and high (blue) fluorescence subpopulation plotted against the time follo\\ ing a ig L glucose pulse for S. cerevisiae FE440 in aerobic gluco chemostuis for frozen cells grown with D= 0.3 h' and D= 0.05 h'. The red and blue colored crt lines are indicating the time when the pulsed glucose is consumed br 0.05 h and 0.3 h respectively. whereas the black line is indicating the time of the glucose pulse.

Comparing with a distinct and constant distribution of fresh cells, particularly for cells growing at a dilution rate above the dilution rate where respiro-fermentative metabolism sets in, a much broader population distribution was seen for frozen cells, which got even more pronounced after the glucose pulse as seen from values for CV, peakwidth and slope of the cdf plot (figure 4 and 5).

Effect of freeze-thaw stress on E. coli cells

Due to the significant influence of freeze-thaw stress on yeast cells, the freezing effect was investigated in E. coli as well. Shake-flasks cultivations were performed and two samples withdrawn for each growth phase resulting in a total of 6 samples throughout the cultivation (sampling time points in relation to growth can be seen in figure 7A). Each sample was divided into three aliquots. one for direct analysis and the other two frozen, one flash-frozen in liquid nitrogen and one more slowly at -80°C. The GFP signal intensity of each sample was subsequently analyzed by flow cytometry.

The fresh cells behaved as expected with a short increase of the GFP signal in early exponential phase followed by a stepwise decrease (figure 7 A and B). After 12 h of growth when the cells had reached stationary phase, the GFP signal decreased further to values only slightly higher than the background level. Subpopulation in terms of GFP expression was not observed during the cultivation (data not shown). it was concluded that freeze-thaw stress only had a minor influence on the GFP intensity in E. co/i, in contrast to what was obser ed for yeast. One probable reason being the higher pH used for the E. co/i cultivations, which would negate the negative effect a low extracellular pH has on :he fluorescence of cells with damaged cell membranes from the freeze-thaw cycle. The GFP synthesis time profile in the batch culture is in agreement with our previous findings that FIS is abundant in the very early log phase of rapidly growing cells. whereafter the pronioter is switched off in late log phase and stationary phase:° in contrasL the corresponding profile of the crlf slope (figure 7 C) showed that there was less influence on the fluorescence distribution for cells in the early and mid exponential pl than in the late exponential and ibe stationary

phases. However, the lower slope may be a consequence of loss of GFP since the fluorescence had fallen back to background evels at this time point. The slope curve peaked in the mid exponential phase, which is in line with the highest growth rate. This can also be seen in the rdfplot that started with a low slope and end with a high slope (figure 7 C). Fluorescence distributions of cells that were frozen at -80°C before analysis, resembled those of the fresh cells as seen from the mean GFP and cdf slope values obtained (figure 7 B, C). Hence, all data presented in the following sections were obtained from samples frozen at -80 °C.
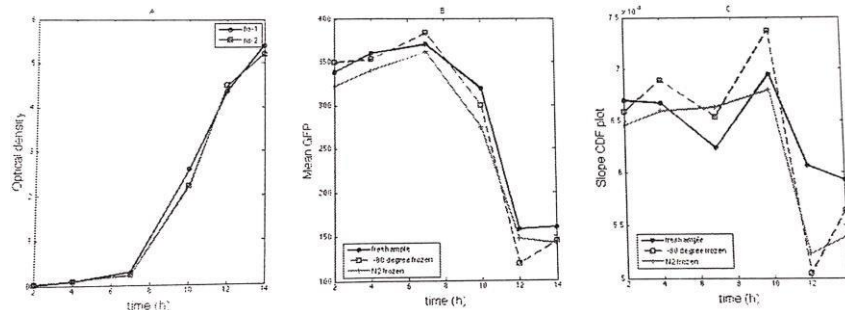
Figure 7. Effects of freeze-thaw stress on GFP signal distribution of bacterial cells. From left to right: growth curve (A), mean GFP (B) and slope of the cdf plot (C). The decrease of GFP fluorescence is connected with increased cdfplot slope. The plots represent average values from two biological replicates.

GFP t1u distribution of E. coli cells at steady-state conditions

The E. co/i reporter strain was subsequently cultivated in continuous mode to inves.igate the effect of growth rate on GFP expression and population heterogeneity. From the FSC-GFP biplots (figure 8) it can be seen that the population spanned over a large range of sizes. which shifted towards higher values with increasing growth rate. An increase of 1 00 and 200 channel numbers was seen for the dilution rates of 0.44 h and 0.53 h. respectively. A similar population distribution was seen for the GFP fluorescence at the different dilution rates (figure 8 and 9). However, a slight shift towards lower fluorescence was observed for the higher growth rates. The mean fluorescence for cells grown at D=0.09 h was 446.4--/-10.8 fluorescence channel numbers, \vl1 cells grown at D0.44 h and D=0.53 h' had a mean fluorescence of 411 .3—-I .0 nd 410.0±-IS, respectively (figure 9, table 5. The low GFP levels found in cefs with relatively high FSC values, might be an effect of these cells undergoing binary fission and hence are diverting cellular resources towards this process. The slope of cdf plots, coefficient of variance (CV) of GFP and the width of the peak for the three dilution rates (figure 9, table 5), showed a somewhat broader population distribution and higher relative standard deviation for the low dilution rate, indicating a higher degree of population heterogeneity at this growth rate. The similar peakwidth for cell grown at the higher growth rates of 0.44 h' and 0.53 h' is lower than D=0.09 h indicating a narrower GFP peak in rapidly growing cells.
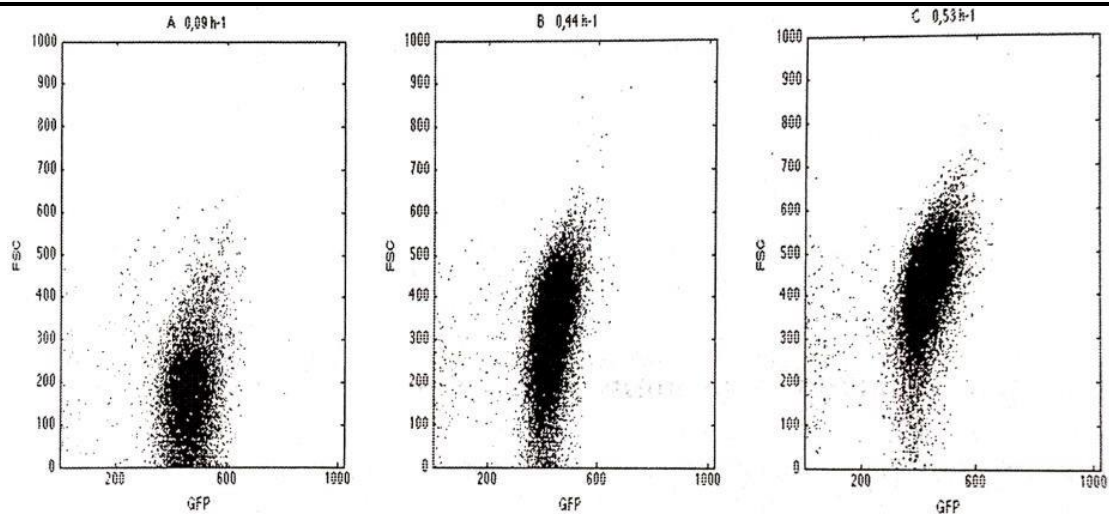
Figure 8. FSC against GFP biplots for E. coli aerobic glucose limited chemostats. From left to right: D0.09 h 0.44 h 0.53 h
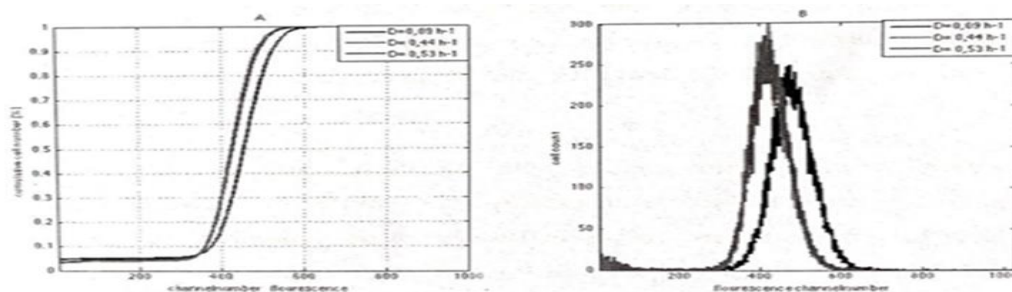
Figure 9. Steady state GFP distributions for E. co/i aerobic glucose limited chemostats. Cdf plot (A) and histogram (B) of D=0.09 h 0.44 h 0.53 h were shown.

Table 5. Quantitative values for measurement of heterogeneity of GFP fluorescence represented as CV of the mean GFP signal, the peakwidth and the slope of the cdf plots including standard deviation at steady state for D=0.09, 0.44 and 0.53 h'.

Parameters	0.09h-1	0.44h-1	0.53h-1
Mean GFP	446.4+/-10.8	411.3+/-1.0	410.9+/-1.5
CV GFP	0.5	0.48	0.5
GFP peakwidth	222.7+/-8.3	198.7+/-2.1	203.5+/-5.1
Slope of cdf plots	0.0063+/-1.94.10-3	0.0073+/-5.11.10-5	0.0070+/-3.49-10-5

Physiological responses of E. coil cells to glucose perturbations

The responses of aerobic, glucose-limited E. co/i cultures to sudden glucose excess was investigated by perturbing the steady-state system with 0.45 g/L glucose pulses. At time point zero, glucose was added and the dynamics of the extracellular metaboljte and intracellular fluorescence levels followed over 3 hours. The glucose pulse ras depleted in 25 mm for D=0.09 h', 35 mm for D=0.44 h and 45 mm for D=0.53 h' (figure 10). No acetate formation was detected at 0.09 h which is consistent with earlier findings. Acetate and pyruvate were already present in the cultivation broth before the perturbation for D=0.53 h due to the overflow metabolism.

However, for both the high dilution rates acetate concentration increased as a consequence of the pulse. The reduction of the acetate level was very slow at D= 0.53 h', as the cells were already growing with overflow metabolism and unable to re assimilate the produced acetate. Acetate in this case was probably just diluted out by the feeding rather than consumed by the cells. Formic acid levels were very low a did not seem to respond to the pulse (data not shown). The CO evolution at D= 0.53 h and D=0.44 h increased slightly after the pulse and a glucose and acetute utilization phase, respectively, could be distinguished from this data.
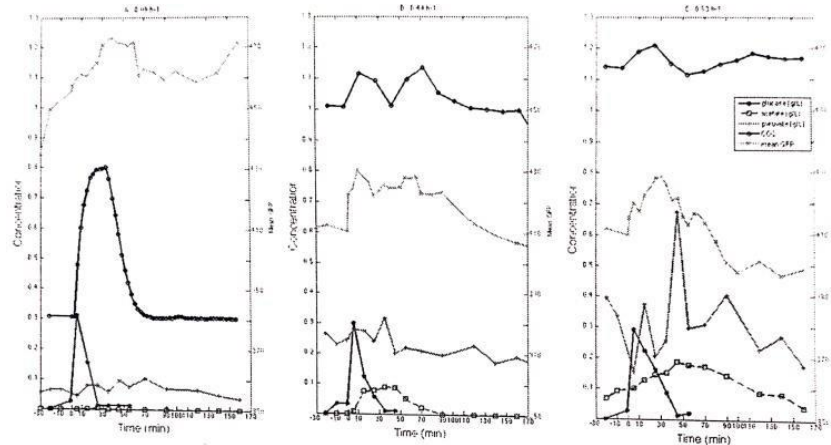
Figure 10. Perturbation profiles of E. coli chemostats after a 0.45 gIL glucose pulse. Profiles for aerobic glucose-limited chemostats, D=0.09 h (A), 0.44 h (B), 0.53 h' (C) (from left to right). Glucose (g/L, blue), acetate (gIL, red), pyruvate (gIL, pink), CO (v/v%, black) and the mean GFP intensity (green).

Influence of the glucose pulse on GFP distribution in E. coli

The pulse seemed to cause a slight shift towards higher fluorescence for all three growth rates, though it could be argued for D0.09 h' as the fluorescence was fluctuating quite a bit in steady state (figure 10). As the pulses abated. the fluorescence returned to lower levels again. No population segregation was found in either of the three dilution rates and the peak shapes remained constant during the pulses (data not shown).

A second distinct acetate re-assimilation phase was seen for D=0.44 h'. where the mean GFP followed the overall trend of the CO evolution. Thus indicating that the reporter strain may have a fast enough response to capture both the glucose and acetate growth phases. Cells growing at the high dilution rate of 0.44 h' and 0. h showed a more dynamic response to the glucose addition th:in the low dilution rate. This can be seen in the slight increase in peakwidth for the faster growing cells. whereas the peakwidth remained almost constant for the low rowth condition (ligure 111 B). No change in peakwidth or CV of the GFP for D=0.0 h', could be Seer after the pulse, indicating that the level of heterogeneity was not affected by the glucose addition. The constant high value of peak width at low cilution rate indicated a broader distribution than the other two dilution rates during the pulse. The generally higher GFP intensity in the continuous culture (figure 10) compared to the batch (figure 7 B) is in agreement with our previous study. The underlying cause might be related to the effect of the metabolic state at exponential growth on the fis promoter or to the fact that environmental conditions change over time during a batch. In particular the decrease in pH and possibly oxygen limitation might affect the overall fluorescence.
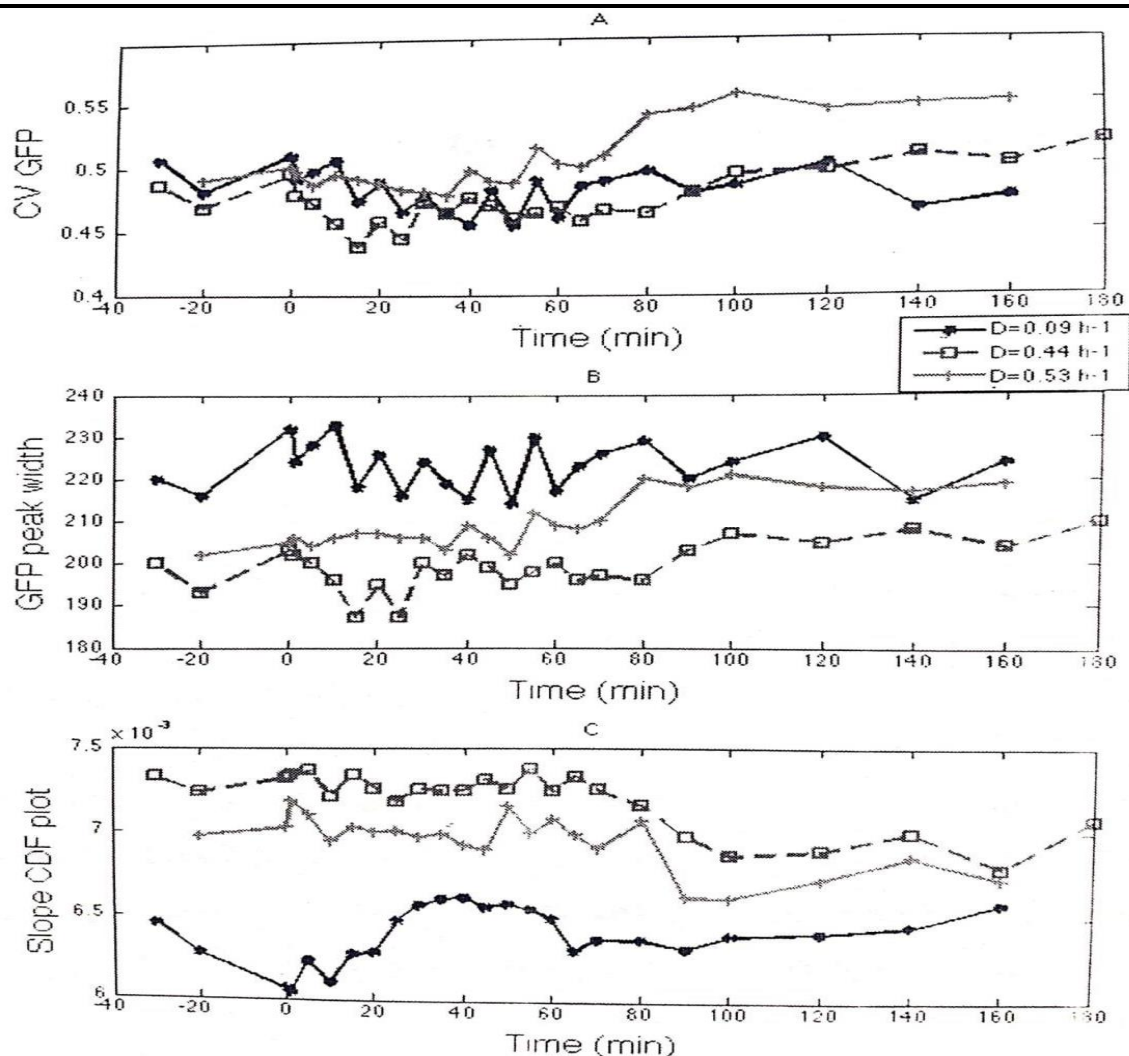
Figure 11. Glucose pulse effects on heterogeneity measured by CV (A). peak width (B), and CDF plot slope (C) of E. co/i chemostats at D= 0.09 h' (green). 0.44 h' (red), 0.53 h' (pink).

Discussion

Cellular heterogeneity is present in all living things from simple bacteria, to eukaryotic and mammalian cells. To explore how cell-to-cell variability correlates with growth rate and glucose limitation, growth reporter strains of S. cerevisiae and E. co/i were grown in steady state at different dilution rates and had their respective population distributions followed over time after the introduction of a glucose pulse.

As suggested earlier, destabilized GFPAAV with a shorter half-life was used in the E. co/i reporter as the employment of stable GFP may have a 'memory' effect on the evolution of gene expression. The rapid turnover of the destabilized GFP facilitates detection of gene down regulations after brief exposures to environmental stimuli as it extinguishes the fluorescent signal when the promoter becomes inactive. Stable GFP on the other hand produces a much brighter signal as it accumulates in the cell, which is an advantage when measuring low expressed genes. In consideration of the different properties and response times in yeast and E. co/i. we constructed a yeast reporter with a stable GFP integrated into a chromosome and an E. co/i reporter with an unstable GFP expressed by a low copy number plasmid.

Quantification of heterogeneity

As unsynchronized microbial cultures are heterogeneous, averaged response values fail to describe the influence of different sub-populations. The currently most used method for single cell analysis is flow

cvtometry, which allows simultaneous quantification of thousands of individual cells per second. The collected data is generally illustrated as single parameter histograirs or in plots w two parameters are correlated, such as biplots. scatterplots or comourplots. Here we present new tools to quantify population heterogeneity using a combination of growth reporter strains and mathematical methods. B using class::al histograms nd biplots (figure 1 as well as figure 4. and 9) and by ca rnc:In GFP fluorescence. general traits of the GFP distribution can be observed. l-lowe\ :r. the selectioa of parameters and divisions of subpopulations are based on subject \e interpretations, which points to the need for more objective standards for the quantification of population heterogeneity. To achieve this the shape of tie distribution values have been enumerated using simple statistical and mathematical functions. which permits the peakwidth and the CV to be used to describe population heterogeneity. The wider the peak the bigger the CV and the more heterogeneous the population. The appearance of sub-populations, shifts over time and differences in peak shape can be illustrated by using a cumulative distribution, which can be quantified via its slope. In conclusion, by using the newly developed parameters a cell population can be objectively described using conventional trend plots over time. These are also easier to comprehend than rows of distribution plots. The challenge, depending on the study, now lies in determining which or what combinations of parameter/s that can reveal relevant information from the torrent of data.

Freezing effect

For practical reasons, it is often convenient to freeze cells before analysis. However, the freeze-thawing procedure is not without consequence and the effects changes with cell type, growth phase. crowth rate and medium composition. In addition, the cooling and thawing rate has a significant influence on the amount of damage inflicted. In general, faster growing cells are more susceptible to freeze-thaw stress, whereas slower growing cells tend to redistribute cellular resources towards stress tolerance thnctions. 3536 We have previously looked into freezing effects in yeast on fluorescence distributions and shape of cells by comparing cells kept on ice versus frozen cells. Whereas there was no observable effect on cell morphology. clear differences w crc seen for the GFP fluorescence including the formation of sub- populations at different fluorescence levels. The faster growing cells in the glucose phase of a batch were mst susceptible. hereas the slower growing cells in the ethanol phase were far more resilient. The underlying cause of the decreasing fluorescence can be two-fild. The loss of GFP molecules through a damaged cell envelope or a decreased f uorescence is probablY due to a pH drop caused by an equalization of the externa. and internal pH via membrane pores/holes. Fluorescent proteins, especiall\ the grc and yellow ariants. arc severely affected by acid shifts close to physiological pH.d The latter being mostly an issue for the yeast cultivations. which were run at the lo ar pH of

5.5 compared to pH of 7.0 used for the E. co/i cultivations. \Ve have prey .ously observed this phenomenon with both freeze-thawed yeast cells and yeast cells permeabilized with Amphotericin B, both displaying a linearly decreasing fluorescence as the e\ternal pH dropped below 7. In contrast. fresh cells did not respond to the pH shifts. In the current study the influence of growth rate on freeze-thaw stress in yeast can be clearly seen in Fig. 2. The slower growing cells at D=0.05 h were unaffected by the freezing procedure whereas the rapidly growing D0.3 h° cells showed a clear drop in fluorescence. In contrast to yeast, we did not see any freeze-thaw effect on the GFP fluorescence in E. co/i during the different phases of

batch growth when comparing fresh cells to cells frozen in either liquid nitrogen or a -80°C freezer. These results indicate pH being the dominant factor in this study.

Influence of steady-state growth rate

The ribosomal RPL22a promoter was initially selected as an indicator of the general growth rate for the construction of the yeast growth reporter strain. Also this growth reporter strain had previously been shown to be growth related in a batch study. In the current study. we did not observe an increase in GFP fluorescence with increasing dilution rate as expected (Fig. 2). Instead, the mean fluorescence level was fairly constant *ith the exception of an increase at the very lowest dilution rate (0.05 h'). However, after normalizing with FSC, which is correlated with cell size in yeast, a clear increasing trend was observed up until D=0.25 h', whereafter the fluorescence/FSC sharply declined. This would indicate that the "expression per cell volurnc increased with growth rate in the respiratory phase. Also, as observed previously in the shifi from respiro-fermentative growth on glucose to respirative growth on ethanol. the particular type of metabolism seems to have a strong influence on fluorescence as indicated by the sharp decline above 0.25 h which marks he onset of respiro-ferrnentative growth.

In this tudy we employed thetI\ promoter as a reporter to visualize shifts in growth conditions of E. co/i. The Fis protein, as an activator of ribosomal promoter, is respon to nutrient availability and is believed to play a global role when E. co/i cells adapt to changes in environmental conditions. Its rapid response to nutrient upshift and downshifks, as well as the fact that its general molecular mechanisms are known makes it an attractive option as a reporter for the study of transient respon in bacterial cultivation processes. The destabilized GFPAA\ variant of GFP cs chosen as it permits the observation of rapid downshifts. however, a plasmid based expression system was deemed necessary to achieve a sufficiently high GFP fluorescence. Here, the steady-state GFP fluorescence was significantly higher at the low dilution rate (0.09 h') (figure 10), which challenged the usefulness of the pFis GFP strain as a pure growth rate reporter. One contributing factor might be that the GFP expression was plasmid based in contrast to the chromosomally integrated yeast reporter, as plasmid counts have previously been shown to decrease at high dilution rates in E. co/i chemostats.

Pulse response comparison between yeast and E. coli.

To follow the dynamics and responses of the reporters to transient changes from glucose limitation to glucose excess and back, I gIL (yeast) and 0.45 gIL (E. co/i) pulses of glucose were introduced to the continuous cultures. Fresh samples from the yeast cultivations showed a slightly shifting fluorescence intensity at the lower dilution rate (D=0.05 h but a constant intensity at the higher dilution rate (0.30 h'). This seems intuitive, as any shift in metabolism after a relief of glucose limitation must be greater for slower growing cells. The frozen cell samples from the low dilution rate cultivations showed comparable shifts to the fresh samples. However, in stark contrast to the fresh cells, the frozen samples from the high dilution rate cultivations displayed significant shifts in response to the pulses (Fig. 5 and 6). This behavior was mirrored by the analysis of the frozen E. co/i cells. At the low dilution rate the E. co/i GFP fluorescence barely shifted with the pulse, whereas as the dilution rate increased, the shifts became more pronounced (Fig. 11). Figure 4 shows how the east population distribution changed after the freeze-thaw cycle. At the low dilution ate there was a slight shift to lower fluorescence and a widening of the peak. In contrast, the high dilution rate

population showed a dramatic peak widening and a shift to a lower fluorescence for a large part of the population. Interestingly, after the introduction of the pulse a larger part of the population maintained the fluorescence. Thus a second mechanism influencing the fluorescence must have been in action for [frozen cells. As discussed above, it is ve11 established that faster growing cells are much more susceptible to freeze-thaw damage than slower growing ones. This can jlso be readily observed here by comparing the fluorescence levels of the frozen and resh yeast cells (Fig. 5A fresh and frozen), where the frozen cell samples from the high dilution rate cultivations had significantly lower fluorescence than the fresh mterpaits.

However, why the pulse seems to protect some of the faster growing cells from freeze-thaw damage is harder to explain. The only thing coimected to the physiology of the cells that can react on the time-scales of the almost instantaneous changes observed is the intra-cellular metabolite levels. One could surmise that the fast growing cells, already primed for over-flow metabolism, quickly absorbed the glucose and perhaps even produced some storage carbohydrates. An intracellular shift in metabolite concentrations might very well affect the physics of the freezing process offering additional protection to the cell membrane. Our results indicate that while the growth rate did not contribute to the expression of our reporter genes in neither yeast nor E. coil, clear up-regulations after the introduction of the glucose pulses were observed. This indicates that the gene regulations of the promoters are primarily responding to environmental shifts rather than growth rate at steady state conditions.

Potential with the reporter strains and future outlook

High throughput single-cell gene expression studies are vital for elucidating the dynamics and contributions of heterogeneous cell populations. In general, the new tools pre here combines growth reporter strains and mathematical methods to explore sub-population dynamics in changing environments. It is difficult to select parameters for the description of fluorescence distribution data. However, changes that previously were only subjectively quantified by comparing classical histograms can be now be objectively quantified and analyzed using this newly developed method.

Acknowledgement

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5.4 Manuscript 4

Studying heterogeneity at single cell level as a way to develop robustness in batch cultures

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Abstract

Microbial cells in batch cultures have traditionally been considered to be uniform populations. However, bacterial heterogeneity occurs and it leads to reduced productivity as suggested in many studies. Our aim is to develop a simple toolbox of combined single cell techniques that will allow rapid assessment of cellular heterogeneity in batch cultures.

Time-resolved cellular heterogeneity profile of Escherichia co strain MG 1655 during batch growth with two carbon sources (glucose and acetate) was studied by the evolution of the destabilized GFP expression with different fluorescent activity stains targeting particular features, using flow cytornetric methods (FCM). Obtained results suggested that the growth depended GFP expression is consistent with the observation of metabolic activity. The protein synthesis (GFP expression) was higher in the acetate growing culture than in the glucose growing culture, suggesting that cells were undergoing distinctive changes trying

to cope with harsh conditions. The Redox Sensor Green (RSG) and Bis-(1 ,3-dibutylbarbituric acid) trimethine oxonol (DiBAC are more reliable as indicators of metabolic activity than other stains used in the study.

Cellular heterogeneity was evident in all the measured parameters, and the developed approach enabled a deeper study of heterogeneity in bacterial batch cultures. Also. the combination of fluorescent probes proved to be an accurate and fast alternative to the employment of biosensors. We would like to highlight the tremendous potential that flow cytornetry has for fast and accurate monitoring of heterogeneity in bioreactors, and eventuall showing us new pathways to enhanced robustness in bioprocesses.

Keywords: Flow cytometry. Biosensor, Heterogeneity. Robustness. Activity Stains

Introduction

The use of living cells for the making of biomass or product formation is the cornerstone of enormously profitable industries. As most cell cultures used in batch cultures are isogenic, these populations have traditionally being regarded as homogenous. However, studies found that local microenvironments induce dynamic genetic, metabolic and physiological cell responses and have been identified as key drivers for the development of cell heterogeneity in bioprocesses." Such an environmental gradient formation effect is unavoidable in large-scale bioreactors and is further magnified by reactor enlargement as a result of deficient mixing, which leads to zones with diverse environmental conditions. Understanding the dynamics of heterogeneity in bioprocess is therefore considered to be a valuable tool for bioprocess optimization since population heterogeneity has a significant impact on product yields, quality and cell viability. Batch culture is predominantly used in many industrial processes due to the simplicity, operability and less contamination of the operation. However, not much is known precisely about the influence of heterogeneity on the process performance.

The different bacterial cell physiological states arise when cells are grown in a closed batch- culture due to constant environment fluctuations, which indicates changes in gene expression and diverse physiological responses to environmental perturbations leading to a physiologically heterogeneous cell population, and consequently, to reduced production productivity as suggested in other studies.

Ordinary bioprocess monitoring often relies on population-based measurements of cell viability and yield production. which is unable to reflect the heterogeneit\ among individual cells within a population.'° Flow cytometry (FC). has been applied to discover and monitor cell-to-cell differences. celluLar mechanisms and re circuits at the single-cell level. With the application of flow cytometrv to microorganisms since late 1970s labeling fluorescently cells. e.g. by staining, has enabled the study of the physiologic and the activity state of bacteria with a higher resolution and these methodologies have been frequently applied to study heterogeneity in batch cultures.

The application of different fluorescent stains synchronically unveils the population heterogeneity and the functional differences between bacterial cells. The tetrazolium dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), is reduced by respiring cells to a water insoluble intracellular red fluorescent formazan and has been commonly used for monitoring the respiratory activity of cells.' The still relatively unused stain, the redox sensor green (RSG) is a vitality stain that becomes fluorescent (green) when altered by bacterial

reductases' and as many of these are part of the electron transport system, this stain has also been employed as an indicator of the respiratory ability of bacteria.' As a counterpart to activity measurements the propidium iodide (P1) dye is commonly used as a membrane integrity indicator. It is a nucleic acid stain that is also membrane impermeable and therefore commonly used to detect cells with disrupted membranes in bacterial populations.' Cells that are not metabolic active and not dead, lack the ability to exchange ions through the membrane, that they have no membrane potential. The bis-(I ,3 -dibarbituric acid)-trimethine oxanol (DiBAC 3)) 20 dye has the ability to enter depolarized cells and while retained inside turns green.

FC also permits to collect information about potential heterogeneities within bioreactors using the microbial populati on itself. The green fluorescent protein gene (gfp) is one of the most well- known and used reporter genes. GFP is a small bioluminescent protein that is used as promoter activity reporter in many bacteria and yeasts cells. not interfering with cell growth. The high degree of stability of the wild type GFP (the fluorescent signal can last for over 24 h might pose a problem in some experiments where transient gene expression is concerned. Non-active, non viable or dormant bacterial cells containing stable GFP would continue to fluoresce or still express the fluorescent signal in the absence of promoter gene expression and the fluorescence would extend over many bacterial generations. Nevertheless, the addition of an AAV-tag to the C-terminal-tail of stable GFP reduces the half-life of GFP from 24 h to 60 min, forming a destabilized form of GFP. in the present study growth reporter was created by fusing destabilized gfp variant (AVV) to the Escherichia co/i ribosomal promoter nmuB PIP2. The best-studied rmB PIP2 promoter when inserted in the plasmid is known to be growth rate regulated and responsive to nutrient starvation and inversely reacts to rRNA gene dosage.

The physiological adaptation of microorganisms to changing environments in batch cultivation is quite distinctive as the degree of heterogeneity in a bioreactor is also dependent on the growth stage of the cells in a batch process. Metabolic versatile E. co/i cells can adapt quickly to the available carbon source such as glucose or acetate, but in the presence of both, cells will preferably consume glucose over acetate. Studies have also shown that the stress response is coupled with cell growth phase in E. co therefore, in this present study we combined the use of CTC, RSG, DiBAC and P1 and FC to investigate heterogeneity in the growth phase of an F. co/i strain with the destabilized GFP reporter system driven by ribosomal promoter rrnB P1P2 growing in a batch bioreactor with either glucose or acetate as carbon source. The state of the individual cells is then reflected in the expression of the reporter protein and fluorescent probes employed and can be followed by flow cytornetry. We hypothesize that this combined approach will reflect the tradeoff between growth and survival along the growth curve. This study aims to cast some light into the intricate relations of single cells and its immediate surrounding environment in a real time basis i bioreactors, which can lead to better monitoring of heterogeneity and ultim became a useful tool in attaining better optimization and robustness of industrial batch cultivations.

Methods Strains

A reporter strain based on the expression of destabilized GFP under the control of rrnB P1P2 promoter, namely. MGl6 was constructed earlier to illustrate the growth dynamic of a bacterial population in

batch cultures. Likewise, the recombinant strain MGI r bearing pGS2OPrrnB was used as the control strain as it did not express GFP. The low copy plasmid pGS2O served as the vector backh

Staining procedure and validation

Five activit\ stains were used in this study: 5-cyano-2,i chloride (CTC) (Sigma-Aldrich, Germany), S BR Green (SYBR) (Invitrogen, USA). Propidiurn iodide (P1) (Sigma-Aldrich. Gennanv), Bis-(l.3-dibutylbarbituric acid) trimethine oxonol (DiBAC (Molecular probes. USA) and Redox Sensor Green (RSG) (Invitrogen, USA). For each staining procedure, I tL of stain was added to 100 .iL of sample suspended in 0.9% of NaC1 saline solution. The final stain's concentrations were lx for SYBR. 0.05 rng/mL for PT, 0.5 mM for CTC and 0.0W mM for both DiBAC and RSG. The CTC staining was incubated during 1 hour at 37°C. The SYBR staining was incubated during 20 mm and DiBAC P1 and RSG for 10 mm at room temperature. The stains were protected from the light during all the procedure.

With the aim to find if staining results vary during sample handling, a simple validation procedure was done. The validation of the staining procedure allowed the optimization of the samples' handling prior to staining and subsequent flow cytornetric analysis. The biosensor MG1655/pGS2OPrrnBGFPAAV was inoculated in shake flasks using 100 mL of a defined minimal media with two different carbon sources, glucose (5 gIL) or acetate (2 g/L). The cultures were grown at 37°C with a continuous stirring of 140 rpm. Samples were taken every hour until the cultures reached the stationary phase for both carbon sources, although the acetate culture sampling started only after 12 h of incubation (after the start of the lag phase). The optical density of the cultures was measured at each time point and the staining procedure was applied to samples immediately, after 30 and 60 mm of ice incubation and after 30 and 60 mm of room temperature incubation. Flow cytometric methods (FCM) were used to detect the growth dependent GFP expression of the biosensor and the different fluorescent activity stains.

Two-way analysis of variances (ANOVA) was applied to the obtained mean fluorescence signals, using the GraphPad Prism 5 (GraphPad, mc, USA). A significance level of 0.05 was employed. Thus. adequate incubation times and temperatures were chosen when no significant differences ($p > 0.05$) were observed between the results obtained immediately after sampling and after the applied incubation conditions. As so, samples for staining with RSG. SYBR green, and P1 and) should be kept on ice until flow cytometric measurements. The samples to be stained with DiBAC were placed at room temperature and after the tenth hour of growth in batch, on ice. The samples to be stained with CTC were kept at room temperature. Proper dilution increases staining efficiency and accuracy, so based on calibration curves relating the 0D and number of events recorded in the flow cytometer, samples with an 0D above 0.2 should be diluted 10 times before staining and further analyzed in the flow cytometer.

Flow cytometry

All bacterial cells were analyzed using a FACSAria III (Becton Dickinson, USA) flow cytometer. The system includes two lasers with minimum laser power of 10 mW with the specific wavelengths of 488 nm and 561 nm. Two scattering channels (FSC and SSC) and two fluorescent detection channels (530/30 nm and 610/20 nm) were used in the analysis. The amplification voltages on the detectors were set based on negative and positive controls (stained, non-stained, viable and non-viable bacterial populations) in a way to

better visualize the populations and were as followed: FSC— 301V; SSC —316 V; 530/30 nm —520 V and 6 10/20 —582 V. The detection thresholds were set in FSC and SSC in order to eliminate background noise.

The flow cytometer (FC) has specific software used in combination with CS&T beads (Cytometer Setup and Tracking beads) (Becton Dickinson, USA) for the automated QA/QC of the machine performance. All results obtained with the FCM were saved as FCS files and analyzed by FlowJo software (Tree star Inc., USA).

Preculture preparation

The inoculurns used to start the batch culture were obtained by a two-steps pre culturing, in the first step, a 100 n overnight culture of the biosensor MG1655/pGS2OPrrnBGFPAAV was obtained, in LB medium, from a fresh culture plate. In the second step. a 10-fold dilution series was made in LB medium from the overnight culture and grown at 37°C for 6 to 8 h on an orbital shaker (160 rpm). The 0D was measure for all the shake flasks and the one with 0.4< 0D 0.6 was picked to inoculate the bioreactors. The medium used in the pre-cultures was supplemented with 25 jtg/mL of chloramphenicol (dissolved in 96% ethanol, stock concentration: 10 mg/mi). Similar procedure was used for preparing the inoculum of the control strain.

Batch cultivation

Batch cultivations of the biosensor MG1655/pGS2OPrrnBGFPAAV strain were grown with 5 g L glucose or 2 gIL acetate as carbon source. Also the control strain MG1655/pGS2OPrrnB was grown in parallel with 5 gIL glucose as carbon source. The growth medium used was a defined mineral medium containing 2 gIL Na 2.468 gIL (NH 0.5 gIL NH 14.6 gIL K 3.6 gIL NaH 1 g/L (NH 50 iL Sigma 204 Antifoam and 0.1 gIL thiamin. After autoclaving, pre-filtrated (0.2 rim) 3 mL MgSO I mL trace metal solution and 25 j.tg/ml chloramphenicol were added into the sterilized medium. The trace metal solution contained 0.5 gIL CaC1 0.18 g/L ZnSO 0.1 gIL MIISO 20.1 gIL Na 16.7 gIL FeC1 0.16 g/L CuSO and 0.18 gIL C0C1 All cultivations were performed using 1-liter bioreactors (Sartorius, B. Braun Biotech International, GmbH, Melsungen, Germany) with a working volume of 1 L. The pH and DOT electrodes (Mettler Toledo, OH, USA) were calibrated according to standard procedures provided by the manufacturer using a two-point calibration (pH 4 and 7 respectively gassing with Oxygen (100%) and with Nitrogen (0%))

The inoculurn (0.4<0D from pre-cultivation was used to inoculate the growth medium to a final OD of 0.005 for both carbon sources. The pH was adjusted and afterwards controlled to pH 7 using 2M NaOH and 2M HC1. Temperature, aeration and stirring were kept constant at 37°C, lv/v/rn and 1000 rpm respectively to avoid oxygen limitation.

Samples for 0 D measurements, high performance liquid chromatography (HPLC), dry weight (DW) and flow cytometry analysis were withdrawn periodically with intervals between 1 .5 and 2 h. Samples for 0D and DW were analyzed directly. whereas filtrate samples for HPLC were kept at -20 °C until measurement. Samples for flo\\ cytometry were kept on ice or at room temperature until analysis (please see "Staining procedure and validation").

OD, DW and HPLC

Growth was monitored at 0D with a Shimadzu UV mini 1240 spectrophotorneter (Shimidzu, Kyoto, Japan). For dry weight measurements 5 ml of the cultivation broth was filtered, washed and dried for 20 mm

at 150W in a microwave, cooled down in desiccators and afterwards weighted on an analytical balance. The concentration of glucose, lactose, acetate, ethanol, glycerol and pyruvate was determined by HPLC (Agilent 1100, Agilent Technologies, CA, USA) with a 300 mm x 7.8 mm Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, CA, USA), refractive index detector (RID Agilent 1200, Agilent Technologies, CA, USA) and UV detector (Agilent 1100, Agilent Technologies, CA, USA) set to 210 nm. The mobile phase was 5 mM H (aq.), temperature 60°C and flow rate 0.6 mL/min. A MS gas analyzer with multiplexer (Thermo Prima SigmaV) was used to monitor the composition of the outgoing gas from the reactors.

Results

In order to study the onset of heterogeneity in axenic batch cultures, a reporter strain (biosensor) based on the expression of destabilized GFP (AAV) under control of the rrnB P1P2 promoter was employed in this study. The use of destabilized gfp gene allows a close follow up monitoring of gene expression by flow cytomeny at either population level or individual cell level. A hoinologue strain not carrying the gfp gene was also constructed as a control strain, in order to assess the biological burden of producing a GFP signal. Both strains were grown in bioreactors with glucose as carbon source and the biosensor reliability was further tested with acetate as a carbon source. The bacterial activity trade-off between cellular growth and survival was examined in both strains by application of various stains targeting the basic metabolic needs of cells.

Cellular growth

The following parameters: glycerol, acetate and glucose. consumed during growth ere measured by HPLC. Also cell mass was measured so that the final carbon balance of the batch cultivations could be calculated in order to test data consistency. The data originated from three different samples, one per reactor (n=3), of which the mean was estimated as well as the standard error. The final summary of yields including standard error showed that the cultivations where glucose was used as carbon source were in balance as shown in table 1. The growth rate and biomass \'Ield of the reporter strain did not differ from the control strain with no GFP expression. icating that GFP expression was not a burden to the cell if using these parameters.

The calculated carbon balance based on the average value of biomass and main produced metabolite concentration was around 1, providing confidence in the accuracy of data as the carbon balance is nearly closed. The data obtained from growth of the biosensor in bioreactors with acetate as carbon source is shown in figure 3. The collected data from all batches performed (n=3) presented a high degree of heterogeneity and for exemplification purposes a set of the most representative data was chosen to be depicted here. When calculating the carbon balance for the reporter strain growing in acetate, around 60-70% of the carbon is missing (data not shown).

Table 1. Yields and carbon balance in the batch cultivation with the respective standard error for biosensor and the control strain with glucose as carbon source (n=3). YSX, YSG, YSAC and YSC are respectively the yields of biomass, glycerol, acetate and carbon dioxide produced.

Yield	Control	Reporter
YSX	0.654575=0.01867	0.695433=0.07329

YSG	0.000457=0.00003	0.000490=0.00006
YSAC	0.313612=0.02335	0.340141=0.01975
YSC	0.082129=0.01133	0.083616=0.01701
Carbon Balance	1.050773=0.00363	1.119679=0.03738

Growth studies in glucose

The control strain showed a typical growth behavior as d by the measurements of the concentrations of the substrates and OD (fg. IA). The glucose was depleted at t=15 h and the acetate switch occurred at I L5 h of growth. The CO consumption increased along the exponential phase and a pea was registered. as expected, just as the strain reached their maximum growth rate (fig. 1 A). The metabolic activity demonstrated by the different staining procedures employed showed an increase of nucleic acids in cells (SYBR green). of respiratory activity (RSG and CTC) and of the number of cells with a compromised cell membrane (P1) immediately before and during the beginning of the exponential phase (fig. 1 B). The staining with DiBAC shows that the number of cells with a depolarized cell membrane increases during the exponential phase and reached a peak at the beginning of the stationary phase. In the present study, a peak of the respiratory activity was observed at the beginning of the exponential phase after which it decreases for the rest of the cultivation period. The measured parameters and data obtained from the activity stains showed larger standard error during the exponential phase as can be observed on figure 1, indicating a high degree of cellular heterogeneity. All the activity indicators depicted slower growth in the late exponential and stationary phases.

Figure 1. Batch cultivation and metabolic activity data along time for the strain MG1655/pGS2OPrrnB (control strain) growing with glucose as carbon source (n=3). The 0D metabolites concentration (g L) and CO % is depicted in (A) the intensities of CTC. RSG, and SYBR green and the percentage of F! and DiBAC4(3) positives cells in (B). Error bars represent standard deviation.

Comparably, the biosensor strain exhibited expected growth behavior in defined mineral media with glucose (fig. 2A). Growth was first supported by glucose. which was exhausted after 10 h and then the acetate switch occurred around the same time.

Stationary phase was reached approximately after 12-14 h. The control strain has a longer lag phase but presents a faster average growth rate (i = 0.72 h') if compared with the biosensor strain (umax = 0.70 h'). The earlier appearance of acetate switch in the control strain (11.5 h) is a reflection of the faster glucose consumption rate and slightly higher growth rate in control strain than the biosensor strain. The CO production increased along with the exponential phase and a peak was registered in end of the exponential growth phase (fig. 2A). The peaks of CO production occurred in different stages of the growth curve for both strains. Likewise, the metabolic activity data registered for PT, RSG and SYBR green also differed in the location of their peaks analogously with the respective growth curve.
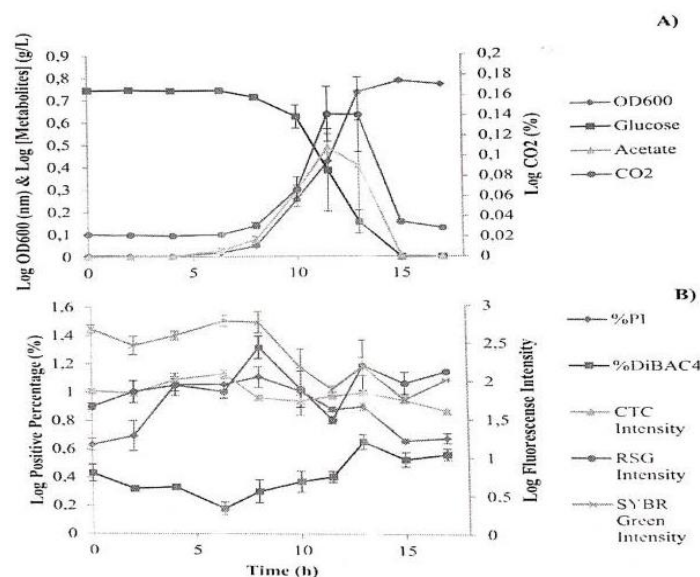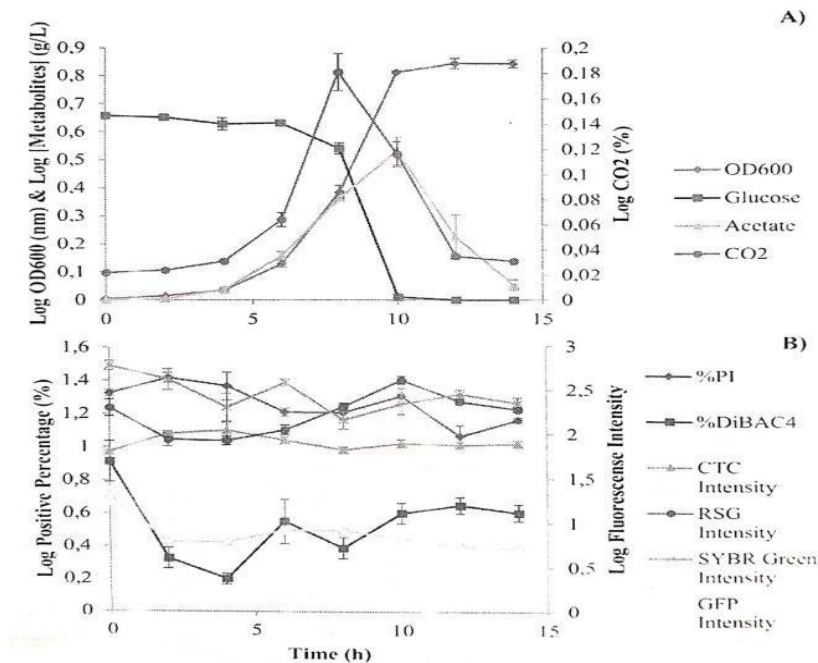

Figure 2. Batch cultivation and metabolic activity data along time for the strain MG1655/pGS2OPrrnBGFPAAV (biosensor strain) growing with glucose as carbon source (n=3). The 0D metaholites concentration (gIL) and C0 is depicted in (A) the ntensities of CTC, RSc and SYBR green and the percentage of P1 and DiBAC4(3) positives cells in (B). Error bars represent standard deviation.

The fraction of P1 and CTC positive cells was immediately increased before and at the very beginning of the exponential phase. Whereas data obtained from the SYBR green and DiBAC showed a peak during the beginning of the exponential phase and a subsequent peak at the onset of the stationary phase, together with the RSG (fig. 2B).

By comparing both strains' growth in glucose (fig. I and 2) and the location of the stain peaks in relation with the growth curve, it was noticed that the biosensor strain had, to a degree, delayed physiological responses when compared with the non- expressing GFP strain. The observed GFP signal obtained via the biosensor correlates well with the exponential phase, indicating that the strain is growing more actively in that phase.

Growth studies in acetate
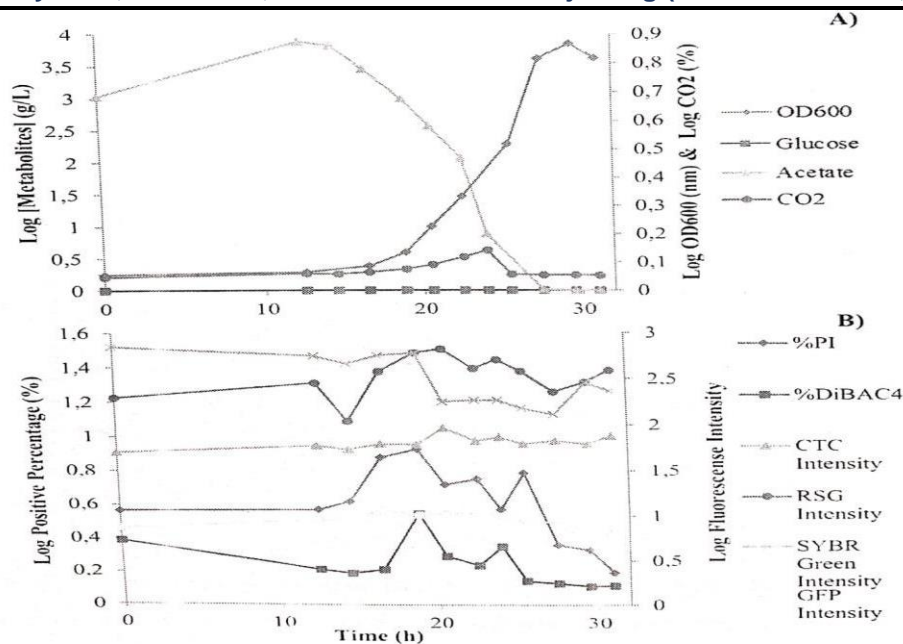
Figure 3. Batch cultivation and metabolic activity data along time for the strain MGi 655/pGS2OPrr IIBGFP AA\ (biosensor strain) growing with acetate as carbon source (n=1). The 0D metabolites concentration (gIL) and CO % is depicted in (A) the intensities of CTC, RSG, and SYBR green and the percentage of P1 and DiBAC4(3) positives cells in (B).

     Comparatively with having glucose as a carbon source, the biosensor strain when growing in acetate has a longer lag phase, a minor average growth rate (= 0.20 h a lower biomass production (OD values) and the CO production is very low all the time with a little peak at the beginning of the exponential phase (fig. 3A). The metabolic activity data retrieved from the staining (fig. 3B) shows that the strain is more active during the very beginning of the exponential phase, as the SYBR green values are higher at that point and the P1, DiBAC and RSG values peak at the same time. The P1 and the DiBAC4(3) have each two coincidental peaks: on the early and on the later exponential phase, after which the percentage of positive bacteria for both stains diminishes. The respiratory activity highlighted by the CTC staining appears to be relatively constant throughout the whole growth. The sub-standard growth conditions of the strain in acetate leads to an increasing number of bacteria, which. are unable to coop with these conditions and become not viable or non-growing (dormant).
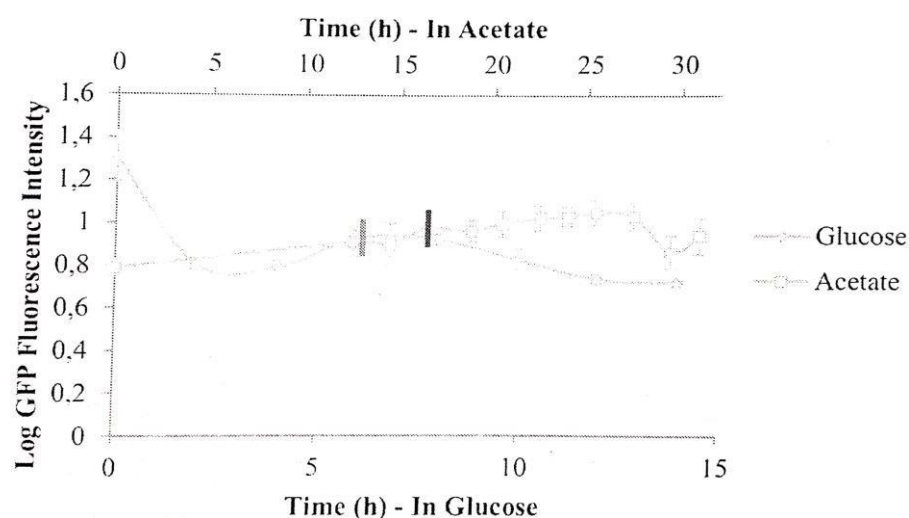
Figure 4. GFP evolution profile of the strain \1G1655/pGS2OPrrnBGFPAAV (biosensor strain) in two carbon sources of glucose and acetate (n=3).

It is known that bacterial populations under stress became asynchronous and contains organisms at all stages of the division cycle leading to a higher level of heterogeneity. The onset of the GFP shows that the strain is actively synthetizing ribosomal RNA during the whole exponential phase as the signal is slightly higher at that point and the signal was approximately 2 times more intense in the acetate medium (fig.4) during the exponential phase indicating a higher production of ribosomal RNA.

Discussion

Our data corroborates earlier studies where heterogeneity in axenic cultures growing in batch is much higher than generally supposed which has significant repercussions for the microbial process analysis (quantitatively and qualitatively). The microbes that were actively metabolizing were detected by flow cytometry-based redox sensing (FCRS) methods, specifically; two redox probes were employed in this study, the CTC and the RSG. In glucose, the control strain appeared to accumulate nucleic acids (SYBR green data) and respire more actively (RSG and CTC data) immediately before the exponential growth phase, showing the cells preparing in high gear for growth. The same parameters for the biosensor strain, with exception of CTC, seam to peak lp.ter during growth. Discrepancies were found between the results obtained with these two stains; the CTC curve is more even out and the signal is less bright when compared with the RSG results. CTC has frequently been used to define the amount of "active" of bacteria in diverse samples, however, there has never been demonstrated a direct correlation between the amount of cells that are actively respiring, growing and dividing and the amount of cells that reduced enough dye to be detected. These facts and the nonexistence of a unified protocol for the application of CTC created a consensus among several researchers that this method lacks great sensibility, it only detects the minimum number of viable cells and obtained results reported in different published studies are hard to compare. RSG, on the other hand, is altered by the active reductases in the cells' and as these are not exclusively present in the electron transport system,' we conclude based on these facts and on our results, that not only is RSG a more reliable but as well is a more sensitive viability and vitality indicator than the CTC.

In contrast to metabolic activity, bacteria with compromised or depolarized membrane are respectively regarded as non-viable or dormant as negatively charged DiBAC cannot penetrate into active cells due to their interior negative charge. In order to access membrane status, P1 and DiBAC were employed in turn to target compromised and depolarized membranes. it was noticed that the percentage of P1 positive cells increases slightly along the growth curve, reaching a peak for the control strain at the beginning of the exponential phase, coinciding with when the cells are accumulating more nucleic acids (SYGR green data). These observations appear to corroborate the existence of false positives as P1 intercalates with any double stranded nucleic acids (DNA and dsRNA). The percentage of DiBAC4(3) positive cells, after an initial stabilization of the culture, are similar in both strains and because the shape of the curves are similar in both cases, we agree that the DiBAC is more suitable as a viable indicator than P1.

The physiological adaptation of E. co/i growing on acetate in batch cultivation is quite distinctive. Growth is inhibited even at very low concentrations,. e.g. 0.5 gIl, and this inhibition becomes very significant in a defined medium. The cellular growth in the acetate batch culture revealed that most of the carbon was

missing (data not shown). By looking at the histograms of the substrate and metabolites obtained by HPLC, an unidentified peak was found, which could be oxaloacetate. A possible reason 'is that the over produced oxaloacetate through the bypass of isocitrate dehydrogenase in acetate grown cultures is central to successful adaption and growth on acetate. Apart from that, the yields of YSX and YSC (biomass and carbon dioxide, respectively) produced and the lower growth rate are found consistent with data retrieved from E. co/i growing on acetate reported previously.

The onset of the GFP signal is coincidental with the exponential phase in both carbon sources and the SYBR green data reveals that in both carbon sources the cells increase the amount of nucleic acids in the beginning of that phase, indicating that cells are preparing to divide. Homologically, the decrease of SYBR intensity is related to lower nucleic acid concentration, since the DNA and RNA concentration on a single cell level basis is lowered after cell division. The measured GFP intensity corresponding to the biosensor on switch indicates that the cells growing in glucose were actively synthesizing ribosomal RNA during the beginning of the exponential phase and that the fraction of active cells was kept at the same level until the end of the exponential phase where the GFP signal was switched off. However when growing on acetate, the GFP expression data shows that the cells are more actively :oclucing proteins even though the growth rate is substantial lower than when growing in glucose. It is known that although starving bacteria have resistance to autolysis, there are mechanisms that overcome this resistance and it has been observed that bacteria are able to disassemble and mark dying cells as response to adverse environmental conditions. As so, the number of inactive cells diminishing at the beginning of the exponential phase for the cultures growing in acetate, lead us to speculate that the harsh conditions select not only for bacteria that grows on acetate but as well for bacteria that are scavenging of cytoplasmic proteins released through autolysis or leakage of not viable or non-growing bacteria, as these ready-made products are more attractive than the acetate. The same thought path can be applied when the cells are growing in glucose to explain why the percentage of P1 positive cells decreases in the end of the exponential phase as this decrease coincides with the drop of the carbon source concentration.

Even if the overall metabolic activity of the strain in all measured parameters conveys the impression of lower growth in acetate, the GFP signal has a higher intensity growing inacetate than when the strain is grown in glucose (fig. 4). This fact is contradictory to the general accepted believe that fast growing cells have a higher number of ribosomal operons resulting in higher synthesis rate of rRNA than cells growing at a slower growth rate. Potentially because the strain's machinery is working harder to cope with the harsh conditions in acetate, growing cells in acetate influences the growth but it enhances bacterial activity, as reflected in more ribosomal activity (fig. 4) and the RSG data (fig. 3). Further investigations are required to fully understand the mechanisms involved in these phenomena.

The physiological state of microorganisms can be affected by a number of environmental factors and consequently tampering with the robustness of bioprocesses. An understanding of the state of the individual cells is thus critical in achieving high process efficiency. Traditional cultivation data collecting reflects the general growth ability of the strains but do not illustrate single cell differences. The developed tool box for this study, using rapid fluorescent staining procedures combined with FCM provided quick infonuation on cellular physiological status changes by crosslinking the data obtained by the measurements of standard

growth parameters, the biosensor GFP expression and the metabolic and physiologic infonnation. This methodology enabled a fast distinction of functionality and metabolic status of cells in the batch cultures during growth. Although this approach was comprehensive, we realized that the single use of the combination of RSG and DiBAC4(3) stains would already reveal more about population heterogeneity than the traditional methods used in the industry to monitor batch cultures. With the given carbon sources, both strains seem to have a period immediately before or after the beginning of the exponential phase where the cells are the most active as if preparing for the growth boom recorded on the exponential phase. This is true for all tested scenarios, except for the biosensor strain in glucose, where this reaction is somewhat delayed when comparing with the growth curve. Leading to the conclusion that biosensor strain pays an energy cost of expressing a gene translated into an alteration of its regular metabolism and physiology, leading to a paradox: The reporter strain is used to monitor growth physiology but at the same time it changes growth physiology.

It is clear that the tested strains prefer glucose as a carbon source to acetate, and that enhanced stress increases the heterogeneity of the population as cells adopt different strategies, exchanging continuously between growth and survival mode, presenting different metabolic and physiological status in each phase of the growth curve.

Acknowledgement

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Chapter 6

Future respective

Escherichia coli is a versatile bacterium that experiences transitions between aerobic and anaerobic metabolism and between nutrient excess and stress as part of its lifestyle. Therefore, it is an ideal system for exploring this capability. Glucose is the most preferred carbon source for bacteria and accordingly changes in glucose availability often have profound consequences in many types of cell gene expression and it ensures

an immediate response in central metabolism. The advantage of using flow cytometry analysis is that it efficiently collects data from loads of cells without any averaging of the signal intensity, therefore, this method was employed throughout this thesis to quantify microbial heterogeneity. The main results were obtained in glucose perturbation experiments followed by the evolution of GFP in high-density cultivation and in chemostat, by physiological correlation between staining parameters and cell growth status. As so, averaged cellular response is re-evaluated by taking the microbial heterogeneity into consideration on single cell level, which enables an accurate behavior assessment of the cells in the population and crucial for the bioprocess control.

Microbial cells have developed mechanisms to respond to environmental changes in a rapid and effective manner, including the rapid reprogramming on the transcription and translation level. Later on the system could be pertubated in an alternative way. e.g. using a different substrate such as acetate, which will yield different responses and therefore gives more important information to capture the distinct stimuli response. On the other hand, the expression of the reporter gene could be examined by RNA-qPCR on the promoter to verify that the GFP level is correlated to the expression or see if there are other things that influence the GFP expression. Further, due to the fact that the plasmid copy number evolves with time and depends on growth phase and growth rate, the variation of copy number might have some effect on the bacterial physiology once the balanced growth is disturbed.

In addition. oxygen limitation is quite often linked with substrate limitation and thus has protound effects on E. co/i physiology. Similar experimental concept can be expanded from glucose to the study of oxygen availability. Perturbation of anaerobic steady-state cultures by providing a limited supply of 02 or the aerobic steady state with perturbation of differed 02 supply might also be interesting. Another, since lower temperature in industrial process is usually used to prevent protein aggregation and maintain the balance between oxygen solubility and growth rate, the heat shock response of cell is thus worth to study during temperature up or down shift.

Future respective

Since a bulk of cells were analyzed simultaneously with flow cytometer, distributions of individual cell behavior within the population (population heterogeneity) can be quantified via the introduced new parameters (manuscript 3) on all the flow data obtained from glucose oscillation process.

Master Chert

Sr.no	Name	Age	Gender	Duration	RBS	FBS	PPBS	HbA1c	E. Coli	colony count
1	Kanwar Lal	60	M	4yrs	217.34	152	260	9.2	yes	1+
2	Shiwani Kanwar	62	F	5yr	152	120	210	8.5	nil	nil
3	Jyoti	47	F	2 yr	192	154	310	10.4	yes	1++
4	Tej Kanwar	70	F	9yrs	168	150	464	10.81	yes	1+++
5	Kavita	70	F	6yrs	192	141	220	8.7	yes	1+
6	Jyoti	45	F	4yrs	195	127	230	8.5	yes	1+
7	Akhilashwar	45	M	6mth	340	161	280	11.8	yes	1++
8	Rakesh	62	M	10yrs	140	110	171	5.9	nil	nil
9	Alphaiz	55	M	8yrs	388	288	355	8.2	yes	1+++
10	Chaina	45	F	1mth	205	131	240	6.5	yes	1+
11	Megha	50	F	3yrs	245	168	230	10.4	yes	1+
12	Ayush	60	M	9yrs	116	90	148	6	nil	nil
13	Krishna	70	M	5yrs	226	180	450	13.8	yes	1++++
14	Laxmi	52	F	6mth	103	161	471	7.8	yes	1++
15	Mahesh	70	M	5yrs	314	212	541	13.4	yes	1++++
16	Kavita	75	F	7yrs	402	246	255	10.6	yes g	1+++
17	Reena	60	F	2yrs	327	102	138	11.4	yes	1+
18	Himat Singh	45	M	4yrs	221	92	137	7.8	yes	1+
19	Shobha	69	F	4yrs	210	125	179	7.6	nil	nil
20	Sangeeta	52	F	3yrs	168	100	151	5.8	nil	nil
21	Deshraj	60	M	20yrs	275	201	332	11.5	yes	1+++
22	Madan Lal	60	M	4yrs	243	110	170	6.2	yes	1+
23	Narendra	60	M	1mth	175	130	210	6.8	yes	1+
24	Shanti Bai	76	F	9yrs	195	161	237	8.7	yes	1+
25	Anita	64	F	3yr	105	131	210	6.2	nil	nil
26	Amrat	45	M	4yrs	288	211	562	18.2	yes	1++++
27	Shahin	60	F	10yrs	118	102	188	6.2	yes	1+
28	Dev Karan	45	M	2yrs	105	78	148	5.6	nil	nil
29	Raghav	65	M	20yrs	159	130	218	7.8	yes	1+
30	Maya	45	F	8yrs	128	116	206	6.2	yes	1+
31	Vidhya	45	F	5mth	168	140	220	7.8	ni	nil

									l	
32	Seema	58	F	10yrs	205	168	260	9.2	yes	1++
33	Bulbul	45	F	new diag.	155	130	226	7.2	yes	1+
34	Salim Ji	60	M	8yrs	178	125	305	5.7	yes	1+
35	Rabiya	63	F	7mth	210	173	241	8.1	yes	1++
36	Hemkanwar	50	F	6yrs	178	132	231	8	yes	1++
37	Raziya	61	F	3yrs	152	129	208	7.3	yes	1+
38	Duli Chand Ji	45	M	1yr	157	110	182	7.6	nil	nil
39	Rukshar	50	M	1yr	204	168	334	8.9	yes	1++
40	Himmat Singh Ji	53	M	8yrs	165	140	280	7.8	yes	1+
41	Raveena	60	F	15yrs	210	130	180	6.5	nil	nil
42	Duli Chand	70	M	6yrs	165	150	205	6.2	nil	nil
43	Sangeeta	45	F	2yr	132	98	204	6.1	nil	nil
44	Sheeba	45	F	4yrs	234	140	298	6.9	yes	1++
45	Ram Lal	54	M	7yrs	282	131	250	6.3	yes	1+
46	Shiv Bai	50	M	8yrs	285	190	380	12	yes	1+++
47	Safique Khan	55	M	2.5yrs	298	210	458	12.8	yes	1+++
48	Safiquik	57	M	3yrs	115	132	205	6	yes	nil
49	Laxman Sahu	60	M	6mth	185	140	239	7.7	yes	1+
50	Seema	50	F	new diag.	195	151	230	8.2	yes	1+
51	Sona	50	F	5yrs	232	151	284	10.4	yes	1++
52	Jamna Bai	60	F	6mth	102	90	146	6.5	nil	nil
53	Shimla	60	F	6yrs	137	97	181	6.6	nil	nil
54	Uma	60	F	12yrs	124	91	168	6.2	nil	nil
55	Seema	52	F	10yrs	180	157	249	8.2	yes	1++
56	Sitara	61	F	5yrs	193	141	239	8.7	yes	1+
57	Rahila	53	F	1yr	375	214	412	13.8	yes	1+++
58	Ramesh Chand Garg	69	M	1mth	335	221	410	12.8	yes	1+++
59	Sushila	62	F	10yr	182	160	304	8.7	yes	1++
60	Abdul Salam	65	M	1yr	212	166	301	9.2	nil	nil
61	Rasid Khan	60	M	2yr	103	92	180	7.6	nil	nil
62	Radheshyam	60	M	5yrs	126	163	280	11	nil	nil
63	Samser Begam	75	F	1yr	158	121	190	6.5	nil	nil
64	Atika Bee	62	F	4.5yrs	280	183	408	9.1	nil	nil
65	Vishnu	80	M	15yrs	203	164	276	7.8	yes	1++
66	Shehnaz	60	F	13yrs	248	171	296	9.2	yes	1++
67	Noram Bai	60	F	1yr	185	128	208	8.2	yes	1+
68	Sunita	70	F	5yrs	285	201	413	11.2	yes	1+++
69	Boori Bai	50	F	1yr	150	168	240	9.3	yes	1+
70	Bhihari	51	M	8yrs	160	140	218	7.3	yes	1+
71	Meena	45	F	2mth	339	282	486	13.6	yes	1+++
72	Sanjida	60	F	new diag.	285	125	247	11.7	yes	1++
73	Akhileshwar	66	M	15yrs	235	151	319	9.9	yes	1++
74	Amna Katun	55	F	8yr	140	90	151	6.6	yes	nil
75	Naval Kishor	65	M	4mth	185	136	241	8.2	yes	1+
76	Pooja	70	F	5yr	135	84	162	6.5	yes	nil
77	Safik Khan	80	M	5yrs	182	12	243	8	yes	1+

						9				
78	Sanjay	45	M	1mth	240	189	396	15.6	yes	1+++
79	Ramesh	60	M	new diag.	452	230	472	16.8	yes	1+++
80	Reena	45	F	5yrs	402	174	431	11.8	yes	1++++
81	Govind	58	M	7yrs	209	155	341	9	yes	1++
82	Suresh Agrwal	58	M	10yrs	195	135	228	8.8	yes	1+
83	Archana	45	F	new diag.	165	139	231	6.9	yes	1+
84	Ghansi Lal	46	M	new diag.	415	201	382	15.8	yes	1++
85	Akina	45	F	6mth	390	240	452	14.6	yes	1++++
86	Raziya	53	F	15.8yrs	334	143	270	12.3	yes	1++
87	Banshilal	52	M	3mth	204	136	261	8.9	nil	nil
88	Hariballabh Soni	52	M	2yrs	205	152	263	9.2	yes	1+
89	Duli Bai	78	F	5mth	388	361	432	13.8	yes	1+++
90	Birdi Lal	55	M	10yrs	285	161	367	11.2	yes	1++
91	Mangla Triwedi	65	M	2yrs	240	90	138	6.8	nil	nil
92	Raziya	45	F	3mth	165	102	180	7.2	nil	nil
93	Mathari	55	F	15yrs	180	93	212	8.2	yes	1+
94	Shila Bai	50	F	2yr	230	140	270	9.2	yes	1+
95	Radhe Shyam	60	F	3yrs	258	161	283	10.2	yes	1++
96	Shiv Lal	50	M	2yrs	387	220	458	15.2	yes	1++
97	Banshi Lal	70	M	3.5yrs	245	158	281	10.2	yes	1+
98	Mathara Bai	45	F	7mth	392	210	403	14.4	yes	1+++
99	Duli Chand	72	M	5yrs	190	110	204	8.7	yes	1+
100	Badri Bai	71	M	6mth	218	123	274	9	yes	1+

ANNEXURE –I

COLLABORATIVE CLINICO-PATHOLOGICAL CASE RECORD SHEET
Places of study:- Department of MICROBIOLOGY , AHMEDABAD
GUJARAT

The aim of this exercise is to define the Indian Priority Pathogen List (IPPL) of antibiotic- resistant bacteria to support and incentivize research and development (R&D) for new antibiotics. We request you expert opinion regardingantibiotic-resistant bacteria in India based on your experience and local data.

SL.NO: OPD   CARD NO: SAMPLE NO: DATE:

- PATIENTS' PARTICULARS:-

I.   NAME:

II.   AGE:

III.   SEX:

IV.   RELIGION:

V.   MOTHER TONGUE:

VI.   ADDRESS:

VII.   PHONE NO:

VIII. POLICE STATION:

IX.   MARITAL STATUS:

X.   EDUCATIONAL STATUS:

XI.   OCCUPATION:

XII.   ECONOMIC STATUS:

ERSONAL HABIT:-

(A) Food	Veg. / Non Veg. (Fresh / Refrigerated / Dried / Smoked / Preserved)
	Spicy Food
(B) Alcohol	Yes / No
	Country Liquor / Foreign Liquor / any other ( )
	Intake: amount in volume:
	How many times/day:
	Duration in years:
(C) Smoking	Yes / No
	Beedi / Cigarette / Ciger / Chuta/Hookli
	Nature direct or reverse
	How many times/day:
	Duration in years:
(D) Khaini / Snuffing	Yes / No
	Place of keeping khaini: labial sulcus/Buccal sulcus
	How many times/day:
	Duration in years:
(E) Chewing	Yes / No
	Pan / Nut / Tobacco/ Pan + Nut / Pan + Tobacco / Pan + Nut + Lime
	/ Pan + Nut + Lime + Tobacco / Others ( )
	How many times/day:
	Duration in years:

	Quid placement: Yes / No
	Duration in years:………………
	Quid in LBM / RBM / Both
	When: Morning / Night / Always

- CHIEF COMPLAINTS:-

- HISTORY OF PRESENT ILLNESS:-

- PAST MEDICAL HISTORY:-

- PAST SURGICAL HISTORY:-

- PAST DENTAL HISTORY:-

- FAMILY HISTORY:-

(a)   General history:-

(b)   History of malignancy:-

CONSENT OF THE PATIENT:-   TAKEN: NOT TAKEN:

- PHYSICAL EXAMINATION OF THE PATIENT:-

- GENERAL SURVEY OF THE PATIENT

i.   BUILT OR STATE OF NUTRITION:

ii.   WEIGHT:

iii.   ATTITUDE:

iv.   CONSCIOUSNESS STATUS:

v.   GAIT:

vi.   FACIES:

vii.   ANAEMIA:

viii. OEDEMA:

ix.   CLUBBING:

x.   SKIN ERUPTION:

- PROVISIONAL DIAGNOSIS

- ROUTINE INVESTIGATIONS

ROUTINE HAEMOGRAM

a.   Haemoglobin%:

b.   T.L.C:

c.   D.L.C:

> Lymphocytes:

> Neutroplils:

> Basophils:

> Eosinophils:

> Monocytes:

d.   E.S.R:

e.   SUGAR:

> PP:

➢   FASTING:

f.   PLATELETS:

URINE EXAMINATION STOOL EXAMINATION:

MICROBIAL ASSAY

LIGHT- MICROSCOPIC FINDINGS(H/P REPORT):-

miRNA ASSAY :   PERFORMED: NOT PERFORMED:

ANY OTHER SPECIAL INVESTIGATIONS:

•   DIFFERENTIAL DIAGNOSIS:

•   CONFIRMATIVE DIAGNOSIS:

•   TREATMENT PLAN:

•   REFERRAL:

Signature of The PhD Student

Signature of The Co-Guide   Signature of Guide

ANNEXURE –II
DEPARTMENT OF MICROBIOLOGY

Sabermati University , Ahmedabad, Gujrat

CONSENT OF THE PATIENT

PLACE :

DATE:

I_____ son   /   wife   /   daughter   of
_____ address   _____   P.S.
_____ do hereby declare that I have been explained the necessity of the physical examination and/ or the surgical procedure required for performing biopsy from the lesion / growth in my orofacial region, or for collection of blood by venupuncture or aspiration of body fluid, as case may be, in the language, I understand, by the doctor concerned, for establishment of diagnosis, for treatment or for research purposes.

I do hereby give my consent in full alertness of my mind for performing the above procedures in my body.

Full signature   Full signature

Impression of the patient   Impression of the patient

Signature of the PhD student   Signature of the Guide/Co- Guide