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OPTIMIZATION AND MODELING OF BACTERIAL PROCESSES

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Abstract

Bifidobacterium longum and *Streptomyces peucetius* are two totally different bacterial species with respect to environmental conditions and process objective. These were used as model organisms in this study. The objective of the *B. longum* process was to produce viable biomass suitable for use as food additive. The objective of the *S. peucetius* process was to produce a medically significant compound toxic to the organism. This study presents the process optimization for both model organisms by the use of experiment designs. The critical down-stream processing stage concerning *B. longum*, freeze-drying, was also optimized using response surface methods. Yeast extract and glucose concentrations together with L-cysteine-HCl·H_2O concentration were found most effective concerning *B. longum* ATCC 15707 growth. The first two were optimized with temperature using a CCC experiment design. Optimal growth and glucose consumption was achieved with temperatures as high as 40°C, a glucose concentration of 20 g l^{-1} and yeast extract concentration of 35 g l^{-1}. With a mixture design, soy peptone, beef extract, bacto peptone and tryptone were identified as growth favoring medium components for *S. peucetius* var. *caesius* N47. A CCF experiment design was constructed for the optimization of environmental conditions concerning *S. peucetius* growth, glucose consumption and ε-rhodomycinone production. Raising the cultivation temperature to 35°C favored growth and glucose consumption, but 30°C was found best for ε-rhodomycinone production. At the optimal temperature, a high aeration control setpoint and a high pH value yielded the best results for all responses. The survival of *B. longum* ATCC 15707 during freeze-drying was found highly temperature dependant. With a proper temperature control strategy during the freeze-drying process over 160% better product activity was achieved with a 50% shorter drying time compared to constant temperature freeze-drying. Kinetic cultivation parameters concerning *S. peucetius* were investigated using both batch and continuous cultivation data. Coefficients for substrate consumption (Y_{XS} 0.536 g g^{-1} and m_S 0.54 mg g^{-1} h^{-1}) and product formation (Y_{PX} 12.99 mg g^{-1} and m_P 1.20 mg g^{-1} h^{-1}) were calculated from chemostat results, and a μ_{max} value of slightly over 0.10 h^{-1} was observed. These parameters were used in the kinetic modeling of the cultivation. A best overall fit from the kinetic modeling was obtained when the logistic equation was used for the modeling of growth. Metabolic flux analysis (MFA) was applied to the chemostat data. This implied that ε-rhodomycinone production is almost linearly dependant on the citric acid cycle (TCA) rate. The kinetic modeling approach gave relatively good simulation results, but could not be used for prediction. This was, however, successfully done using neural networks.

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Asiasanat: aineenvaihduntavuoanalyysi, *Bifidobacterium longum*, ε-rodomysinoni, kasvatus, kemostaatti, keskuskomposiittikoesuunnitelma, mallinnus, neuroverkko, optimointi, osafaktorikoesuunnitelma, rakenteeton kineettinen malli, seoskoesuunnitelma, *Streptomyces peucetius* var. *caesius*

Tiivistelmä

Bifidobacterium longum ja *Streptomyces peucetius* ovat ympäristövaatimuksiltaan ja prosessitavoitteiltaan kaksi täysin erilaista bakteeria, joita käytettiin tutkimuksen malliorganismeina. *B. longum* prosessin tavoitteena oli tuottaa elintarvikelisäaineeksi kelpaavaa elävää biomassaa. *S. peucetius* prosessin tavoite oli tuottaa lääketieteellisesti merkittävää ainetta, joka on myrkyllinen organismille itselleen. Tässä työssä optimoitiin molempien malliorganismien kasvatusprosessi koesuunnitelmien avulla. *B. longum* -bakteerin säilytyksen kannalta merkittävin prosessivaihe, kylmäkuivaus, optimoitiin myös vastepintamallitusta apuna käyttäen. Hiivauutteen, glukoosin ja L-kysteiini-HCl·H_2O:n pitoisuudet kasvatusalustassa vaikuttivat eniten *B. longum* ATCC 15707 -bakteerikannan kasvuun. Näistä kaksi ensimmäistä optimoitiin lämpötilan kanssa CCC koesuunnitelman avulla. Kasvu ja glukoosin kulutus olivat optimaalisia jopa 40 ^{o}C lämpötilassa, 20 g l^{-1} glukoosipitoisuudella ja 35 g l^{-1} hiivauutepitoisuudella. Soijapeptoni, lihauute, baktopeptoni ja tryptoni olivat seoskoesuunnitelman mukaan parhaiten *S. peucetius* var. *caesius* N47 -bakteerin kasvua edistäviä alustakomponentteja. *S. peucetius* -bakteerin kasvu, glukoosin kulutus ja ε-rodomysinonin tuotto optimoitiin ympäristömuuttujien suhteen CCF-koesuunnitelman avulla. Korkeammat kasvatuslämpötilat nopeuttivat kasvua ja glukoosin kulutusta, mutta 30 ^{o}C oli paras lämpötila ε-rodomysinonin tuottoon. Optimilämpötilassa sekä korkea ilmastus että pH paransivat kaikkia mitattuja parametreja. *B. longum* ATCC 15707 -bakteerin selviytyminen kylmäkuivauksesta oli tutkimuksen mukaan erittäin lämpötilariippuvaista. Sopivaa lämpötilan säätöstrategiaa käyttämällä saatiin 160 % parempi tuoteaktiivisuus 50 % lyhyemmässä ajassa kuin vakiolämpötilassa suoritetulla kuivauksella. *S. peucetius* -bakteerin kineettiset kasvuparametrit selvitettiin panos- ja jatkuvatoimisista kasvatuksista saadun datan avulla. Jatkuvatoimisten kasvatusten avulla selvitettiin substraatin kulutuksen (Y_{XS} 0,536 g g^{-1} ja m_S 0,54 mg g^{-1} h^{-1}) ja tuotteen muodostuksen (Y_{PX} 12,99 mg g^{-1} ja m_P 1,20 mg g^{-1} h^{-1}) vakiot sekä μ_{max}, joka oli hiukan yli 0,10 h^{-1}. Näitä parametreja käytettiin panoskasvatusten kineettiseen mallinnukseen. Paras mallinnustulos kineettisillä malleilla saatiin logistista kasvuyhtälöä käyttämällä. Jatkuvatoimisten kasvatusten dataan kokeiltiin myös aineenvaihduntavuoanalyysiä (MFA). Tämän perusteella ε-rodomysinonin tuotto riippuu lähes lineaarisesti sitraattisyklin (TCA) nopeudesta. Kineettisellä mallinnuksella päästiin melko hyviin simulointituloksiin, mutta menetelmää ei voinut käyttää mielivaltaisen panoksen ennustamiseen. Tämä saavutettiin kuitenkin neuroverkkomallinnuksen avulla.

Preface

The work was carried out in the Laboratory of Bioprocess Engineering, Department of Chemical Technology, Helsinki University of Technology, during the years 2002-2006.

First, I must thank Professor Matti Leisola for the supervision of this thesis, encouraging me to study the field of biotechnology and the opportunity to work in his laboratory. I would like to express my warmest thanks to Dr. Tero Eerikäinen for the enthusiastic and ever helping manner in leading the research project, for giving the final push to start my doctoral studies and for the constant encouragement in both research and study. I would also like to thank the research group members, Erkka Meskanen for motivation, Ulla Moilanen for extensive help in the chemostat studies, Kalle Salonen for innovative ideas in making work in the laboratory easier, and Tero Jokinen for help with Matlab.

I thank the personnel of the Laboratory of Bioprocess Engineering for an enjoyable working environment. Especially, I am grateful to Seppo Jääskeläinen for all the technical assistance, Esa Uosukainen for his efforts in making everything run smoothly, Dr. Ossi Pastinen for help in the "impossible" questions, and Marjaana Rytelä for patience in my analysis problems. Piia Appelqvist, Johanna Aura, Dr. Fred Fenel, Miia Helanto, Johanna Karimäki, Auli Murrola, Dr. Antti Nyyssölä, Anne Pihlajaniemi, Kirsti Pitkänen, Heidi Salo, Dr. Harri Santa, Dr. Salem Shamekh, Noora Sirén, Dr. Ossi Turunen, Erika Winquist, Veera Virtanen, Dr. Antti Vuolanto and Antti-Jussi Zitting are all acknowledged for help and good conversations. In addition, I express my gratitude to Professor Tom Granström for the advice on metabolic flux analysis, and Dr. Niklas von Weymarn for never being too busy to help.

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Finally, I wish to thank my family and friends for support and criticism.

"The man in black fled across the desert, and the gunslinger followed."

Tuusula, July 14, 2006

Kristiina Kiviharju

List of publications

I Kiviharju K., Leisola M., and Eerikäinen T., Optimization of a *Bifidobacterium longum* production process, *J. Biotechnol*. **117** (2005) 299-308.

II Kiviharju, K., Leisola, M., and Eerikäinen, T., Optimization of *Streptomyces peucetius* var. *caesius* N47 cultivation and ε-rhodomycinone production using experimental designs and response surface methods, *J. Ind. Microbiol. Biotechnol.* **31** (2004) 475-481.

III Kiviharju, K., Moilanen, U., Leisola, M., and Eerikäinen, T., A chemostat study of *Streptomyces peucetius* var. *caesius* N47, *Appl. Microbiol. Biotechnol.* (*In Press*).

IV Kiviharju, K., Salonen, K., Leisola, M., and Eerikäinen, T., Modeling and simulation of *Streptomyces peucetius* var. *caesius* N47 cultivation and ε-rhodomycinone production with kinetic equations and neural networks, *J. Biotechnol.* (*In Press*).

The author has been responsible for designing and carrying out all the experiments. Ulla Moilanen helped with the chemostat cultivations for publication III. For publication IV, Kalle Salonen developed the optimization protocol for the kinetic model parameters and Tero Eerikäinen conducted the neural network analysis and wrote the first versions of the manuscripts concerning them. Other authors helped in revising the text.

Abbreviations and symbols

a	activity of bacterial product (cfu g^{-1})
acp	acyl carrier protein
ACN	acetonitrile
ATP	adenosine triphosphate
CCB	Box-Benkhen central composite design
CCC	central composite circumscribed design
CCF	central composite face-centered design
cfu	colony forming units
C_G	glucose concentration in experiment designs (g l^{-1})
CoA	coenzyme-A
C_Y	yeast extract concentration in experiment designs (g l^{-1})
D	chemostat dilution rate (h^{-1})
DCM	dichloromethane
dExp	absorbance change during exponential phase in Bioscreen experiments
DNA	deoxyribonucleic acid
DO	dissolved oxygen (%)
EMP	Embden-Meyerhof-Parnas pathway
F6PPK	fructose-6-phosphate phosphoketolase
HPLC	high performance liquid chromatography
k	Teissier equation kinetic parameter (g l^{-1})
K_C	Contois equation kinetic parameter (g g^{-1})
K_M	Monod equation kinetic parameter (g l^{-1})
Lag	lag phase length in Bioscreen experiments
m	weight (g)
MFA	metabolic flux analysis
MRSC	MRS medium supplemented with 1 g l^{-1} L-cysteine-HCl·H_2O
m_S	maintenance coefficient (g g^{-1} h^{-1})
NAD(P)(H)	nicotinamide adenine dinucleotide (phosphate)(reduced)
NHAACP	aromatized form of the cyclic reduced 9-hydroxy-3,5,7,11,13,15,17,19-nonaoxo-henicosanoyl-[acp]
NHACP	9-hydroxy-3,5,7,11,13,15,17,19-nonaoxo-henicosanoyl-[acp]
NHCACP	cyclic reduced form of 9-hydroxy-3,5,7,11,13,15,17,19-nonaoxo-henicosanoyl-[acp]
OD	optical density
P	ε-rhodomycinone concentration (mg l^{-1})
p_+	maximum ε-rhodomycinone production rate in the aerated CCF experiments (mg l^{-1} h^{-1})
p'-	maximum ε-rhodomycinone production rate in the modified non-aerated CCF experiments (mg l^{-1} h^{-1})
P_{max}	ε-rhodomycinone saturation constant (mg l^{-1})
PPP	pentose phosphate pathway
Q^2	coefficient of model prediction
q_{CO2}	specific CO_2 production rate (g g^{-1} h^{-1})

Q_G	specific glucose consumption rate (g g^{-1} h^{-1})
Q_X	biomass production rate (g l^{-1} h^{-1})
$Q_{\varepsilon r}$	specific ε-rhodomycinone production rate (mg g^{-1} h^{-1})
R^2	coefficient of determination
r_e	ε-rhodomycinone production rate (mg l^{-1} h^{-1})
r_G	maximum glucose consumption rate (g l^{-1} h^{-1})
RNA	ribonucleic acid
RQ	respiratory quotient
S	substrate concentration (g l^{-1})
t	time (h)
T	temperature (^oC, K)
t_1	phase 1 length in freeze-drying experiments (h)
t_2	phase 2 length in freeze-drying experiments (h)
t_3	phase 3 length in freeze-drying experiments (h)
TCA	citric acid cycle
TFA	trifluoroacetic acid
t_G	relative glucose consumption time (h g^{-1} l)
V_{max}	maximum specific ε-rhodomycinone production rate (mg g^{-1} h^{-1})
X	biomass concentration (g l^{-1})
X_{max}	biomass saturation constant (g l^{-1})
Y_{CS}	yield of CO_2 from glucose (g g^{-1})
Y_{XS}	biomass yield from substrate (g g^{-1})
α	product formation effect on biomass (g g^{-1})
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})

Contents

1 Introduction

Bacteria have been used for the benefit of man for thousands of years. Applications vary from dairy to the production of useful compounds and purification processes. The last 100 years have brought mathematical methods closer to the subject. Models are being built to describe both the reactor and the reaction. Nowadays even the microbial metabolism, signaling and control are being modeled as computing power increases.

The literature part of this work introduces the methods and organisms used in the experimental part. Statistical methods and experiment design are covered briefly with the critical coefficients relevant in the interpretation of the results. Process modeling is reviewed with regard to metabolic flux analysis (MFA), unstructured kinetic models and neural network models. The model organisms *Bifidobacterium longum* and *Streptomyces peucetius* var. *caesius* are introduced, and the work related to the methods used in this study and both genera are briefly reviewed.

The experimental part of this work deals with the use of different screening and optimization methods, namely the mixture design, factorial designs and central composite designs combined with statistical analysis and response surface methods. This part of the work includes both model organisms. The different modeling aspects of the work concern *S. peucetius*, which is the less studied and more complicated modeling subject. The modeling task is approached through continuous cultivation studies combined with MFA, and batch cultivation studies combined with unstructured kinetic models and neural network models.

1.1 Statistical methods in experiment design and evaluation

Statistical process parameter evaluation, experiment design and process optimization have been successfully used in many areas of research. Microbial processes are biological, and

thus can contain relatively large amounts of natural variation. The reaction networks associated with the use of microbes are complex, and many things can have effects on different parts of the networks. Cross effects are possible, even probable, when this amount of complexity is in question. Rational experiment design and statistical evaluation of the results can increase knowledge on the reliability of the information obtained during an experiment set. Furthermore, the amount of experiments required for reliable process optimization can be reduced using experiment design.

1.1.1 Mixture design

One of the first concerns in microbial processes is the rapid definition of a suitable cultivation medium. There are usually many different components to choose from, and a quick and reliable screen can come in handy. Mixture designs are a popular simplex optimization technique for the evaluation of the effects of different medium components, especially in the pharmaceutical industry (Gabrielsson *et al.*, 2002). The principle is to keep the sum of component proportions as 100%. Thus the component concentrations cannot be independently changed (Gabrielsson *et al.*, 2002). The most common are the simplex lattice and simplex centroid designs (Montgomery, 1997).

1.1.2 Factorial design

A good way to evaluate the relative effects of multiple variables is a factorial design. A general two-level factorial design is a 2^k design (Montgomery, 1997). As the number of factors k increases, the number of experiments required for the complete analysis of the design increases rapidly. With 8 factors (2^8 design) the total number of experiments is 256. To save usually scarce experimental resources, fractional factorial designs are often used, when the number of experiments exceeds the resources available. The amount of data attainable from the experiment set is naturally reduced, when fractioning is applied (Montgomery, 1997). To study the main effects of 8 factors, a 2^{8-4} design is adequate, requiring only 16 experiments. The resolution of the fractional factorial design tells about aliasing in the design (Montgomery, 1997). This means whether or not certain effects are hidden under others in the design. A resolution IV design (2^{8-4}_{IV}) is such that no main effect is aliased with another main effect or a two-factor interaction. The results of fractional

factorial designs are evaluated as effect direction and significance. Full factorial design results can be evaluated as response surfaces.

1.1.3 Central composite designs

When critical process parameters are found, they can be further optimized using central composite designs. Central composite designs are popular types of designs for fitting second order models (Montgomery, 1997). The Box-Benkhen design (CCB) is a good example of spherical designs, and the central composite face-centered (CCF) and central composite circumscribed (CCC) of cuboidial designs. The CCF and CCC theory is presented in Figure 1. In a face-centered design 2 center points are enough for the validity of the model, but 3 are often used in order to get a good estimate of the error. Variable transformations are often done to obtain linearity of both factors and responses (Montgomery, 1997). For example studying temperature effects on biological systems often requires a transformation to T^{-1} because of the nature of the kinetic reactions related (Watier *et al.*, 1996). For the same reason rates as responses often require logarithmic transformation. The results of central composite designs are evaluated as response surfaces.

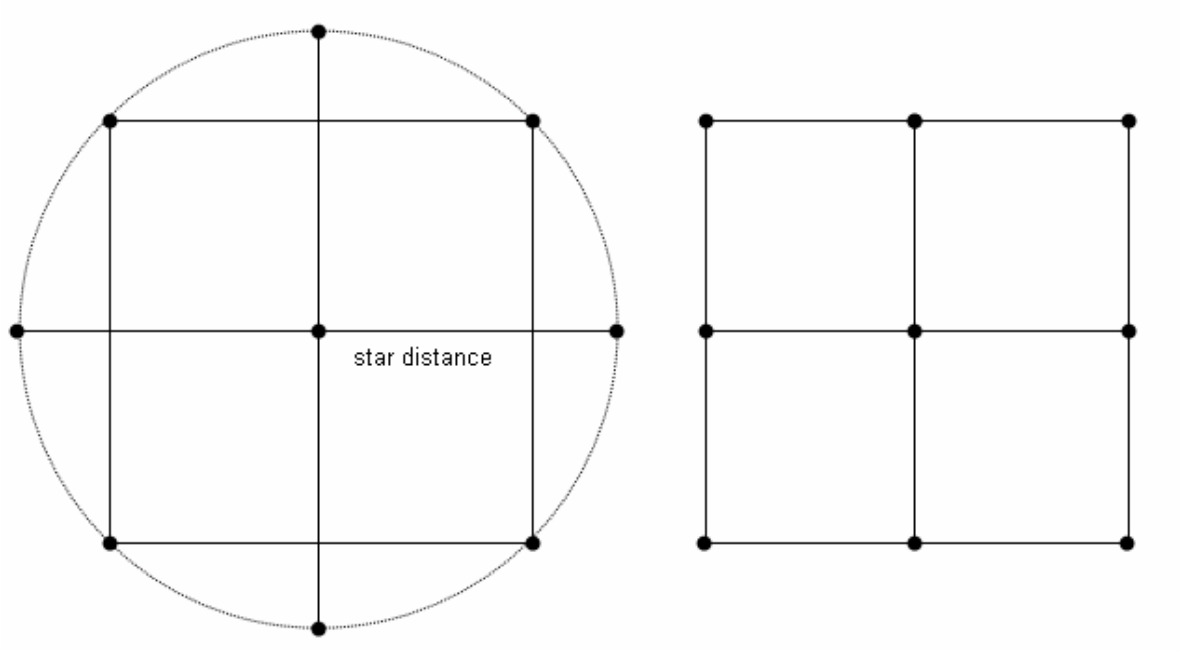

Figure 1. CCC (on the left) and CCF (on the right) experiment design theory with 2 variables. The dots represent experiment points and the squares the modeling area.

1.1.4 Model evaluation

When evaluating the results, it is critical to understand how useful the data is and how good the obtained model is. First, the raw data is evaluated in order to find crude experimental errors. Then, the model for the data and responses is calculated (Gabrielsson *et al.*, 2002). This is usually done by fitting a regression model to the experiment design. There are several ways to evaluate the calculated model, the three coefficients introduced here being the most popular ones. One of the most used statistical coefficients is the P value. This probability to 0-hypothesis tells what the probability is that there is no model-explained variation in the results (Montgomery, 1997). The P value is used for the evaluation of model significance. A very significant model has a P value below 0.001, a significant model below 0.01 and an almost significant model below 0.05. The proportion of variability in the data is shown by the coefficient of determination (R^2), which measures the variance explained by the model (Gabrielsson *et al.*, 2002). The coefficient of model prediction (Q^2) explains the amount of variance predicted by the model (Gabrielsson *et al.*, 2002; Montgomery, 1997). Good values for R^2 are 0.8-0.9 and for Q^2 over 0.5 (Gabrielsson *et al.*, 2002), and their difference 0.2-0.3 (Eriksson *et al.*, 2000). The calculated model is used for evaluation of the results as a response surface plot, where the model is plotted against two or three of the most significant variables (Gabrielsson *et al.*, 2002).

1.2 Bioprocess modeling and control

Modeling of biological processes has always been somewhat challenging. The easiest modeling concepts presume ideal mixing, which is rarely, if ever, achieved. The asepticity requirements make measurements from the reactor non-trivial. Cellular processes, mass transfer and control aspects make the modeling task appear even more daunting, and thus it should carefully be considered, what really needs to be modeled given the specific problem. Different tools have been developed in recent years for different modeling needs. Some concentrate on the reactor, some on reaction kinetics (Eungdamrong and Iyengar, 2004), and some on system biology: metabolomics, proteomics and genomics (Goesmann *et al.*,

2003; Klein *et al.*, 2002; Lemerle *et al.*, 2005). Here, brief overviews on metabolic modeling, kinetic process models and neural network models are given.

1.2.1 Metabolic flux analysis (MFA)

Metabolic flux analysis (MFA) takes into account all cellular reactions from transport to intracellular reactions. Transport is considered to be the transfer reactions of extracellular metabolites through cell wall and membrane structures into the cell as well as the transport through intracellular membrane structures. Intracellular reactions include anabolic and catabolic reactions and secondary metabolism. Macromolecules like DNA, RNA, proteins and biomass associated carbohydrates are produced via anabolic reactions. Catabolic reactions produce ATP and NADPH, which are the energy currency of cells. The major catabolic pathways are the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PPP) pathway, and the citric acid cycle (TCA). In addition to ATP production, the TCA is responsible for the production of biomass building blocks consumed by the anabolic reactions (Nielsen and Villadsen, 1994).

Generally, flux analysis considers the reaction stoichiometry in matrix form. A good description on constructing the network and matrix is given by Granström (2002). Extracellular components are the easiest to measure, and the number of measurements made vs. the properties of the stoichiometric matrix is an important factor when examining the metabolic network. If the rank (number of linearly independent rows or columns) of the stoichiometric matrix is smaller than the matrix dimensions, the network is redundant. In addition, if the number of the measured reaction rates is smaller than the degrees of freedom in the network, the system is underdetermined. Underdetermined systems are analyzed using flux optimization. If the rank of the stoichiometric matrix is one of the matrix dimensions, the network is non-redundant. In addition, if the number of measured reaction rates exceeds the degrees of freedom in the network, the system is overdetermined. Exactly determined and overdetermined networks are analyzed using flux balancing. In overdetermined systems, the extra information obtained in the excess measurements can be used in gross measurement error detection or improvement of result accuracy (Nielsen and Villadsen, 1994).

1.2.2 Kinetic models

Generally, kinetic models are experimentally derived mathematical formulas that fit the cultivation data reasonably well. The easiest kinetic modeling technique is the application of an unstructured kinetic model. The kinetics can be linear or non-linear, single-phase or multiphase (Eungdamrong and Iyengar, 2004; Mitchell *et al.*, 2004). Linear kinetic models include constant rate and first order kinetics. Non-linear kinetic models comprise exponential, logistic, second order and other defined functions (Eungdamrong and Iyengar, 2004; Maurer and Rittmann, 2004; Mitchell *et al.*, 2004).

The most popular defined kinetic model function has undoubtedly been the Monod model, which contains two parameters that define the relation of growth and substrate consumption. Contois and Moser presented modifications to the Monod model. The Contois theory was based on a possible growth inhibition by biomass itself, which has later been doubted (Nielsen and Villadsen, 1994). Altogether different formulas were presented by Teissier and Blackman. The logistic expression has also been successfully used in growth estimations. The Monod model has also been extended to substrate and product inhibitions (Nielsen and Villadsen, 1994). These, however, have more parameters and require more computing power to fit appropriately.

Complex rate law models have been used in the simulation of entire metabolic pathways (Curto *et al.*, 1995). Kinetic models have even been applied to the cultivation lag and death phases as well as to particle interactions (Mitchell *et al.*, 2004; Swinnen *et al.*, 2004), but these are starting to resemble structured models. Temperature and pH correlations have been introduced to the kinetic parameters (Maurer and Rittmann, 2004; Mitchell *et al.*, 2004; Nielsen and Villadsen, 1994).

1.2.3 Neural networks

Modeling and simulation can also be conducted with no prior knowledge of the process variable dependencies by using artificial neural networks (Eerikäinen *et al.*, 1994). An example of this direct approach is presented in Figure 2. Artificial neural networks are good

in non-linear problems and are trained iteratively from known input/output vector pairs. Neural networks are typically applied in estimation and multi-step prediction problems, but can also be used as controllers, either directly or as a parameter adjuster for a conventional controller. In biotechnological processes, neural networks can be applied as soft sensors to predict the cell concentration in batch fermentations (Linko *et al.*, 1994) or as on-line variable estimators for e.g. viscosity and penicillin concentrations in penicillin production (Arauzo-Bravo *et al.*, 2004).

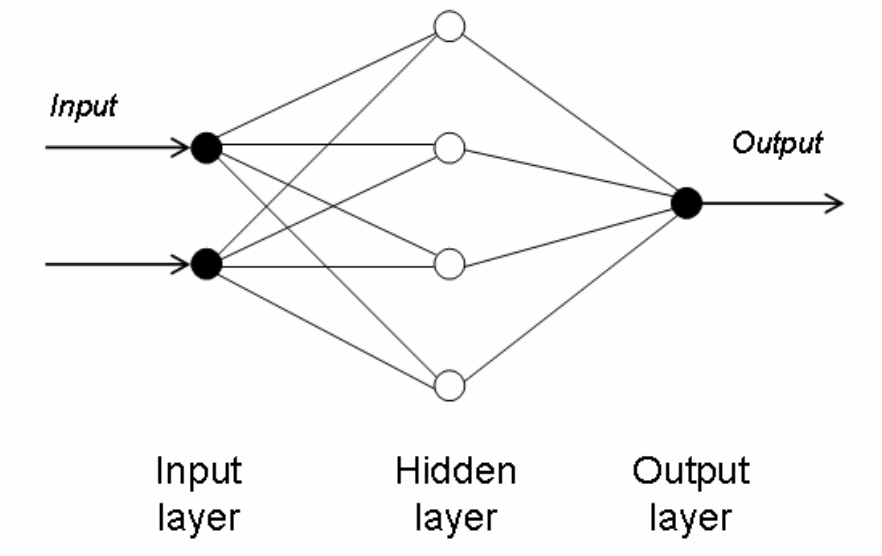

Figure 2. Example of a neural network. Two input layer neurons are connected to 4 hidden layer neurons, which are connected to one output layer neuron. The hidden layer performs the calculations according to the transformation function and weights.

One of the most widely used neural networks is the fully connected feed-forward network. The network is composed of nodes arranged into three layers, where every neuron is connected to each neuron in the next forward layer. Input layer neurons perform no computations, but only transmit the coded (normalized) values to each connected neuron in the hidden layer. Every input and output node corresponds to a measured input or output. Hidden and output layer neurons have a non-linear transfer function.

Prior to use, the network needs to be trained. Here is a short review of the widely used back propagation training algorithm (Rumelhart *et al.*, 1986; Werbos, 1974). The method is an

iterative gradient algorithm, which was designed to minimize the mean square error between the actual output of a multilayer feed-forward network and the desired output. The training procedure is highly repetitive. Example input/output vector pairs are introduced to the network several times, and the neuron weight factors are updated by back propagation after each introduction. The algorithm is briefly described in the following stages.

1) Initialize all weights to small random values.
2) Present normalized input values.
3) Calculate actual outputs from inputs used.
4) Calculate global and local errors between desired and actual output values.
5) Calculate delta weight values recursively from output local errors.
6) Update all weights by adding delta weights to the corresponding previous weights.
7) Repeat by going to step 2 until global error reaches the desired level.

A more detailed description of the back propagation method was introduced by Werbos (1974).

1.3 Bifidobacteria

Bifidobacteria were first discovered in 1899 (Ballongue, 1998; Leahy *et al.*, 2005) and they were initially considered lactic acid bacteria (Axelsson, 1998). The first reference to *Bifidobacterium* was made in 1924 (Ballongue, 1998; Leahy *et al.*, 2005). Until the 1960's there was still uncertainty about the correct classification of this species, as the distinction was made by morphological characterization. Bifidobacteria were confused with *Actinomycetaceae*, *Arthrobacter*, *Corynebacterium*, *Lactobacillus*, and *Propionibacterium* (Axelsson, 1998; Ballongue, 1998). The discovery of the key enzyme in the *Bifidobacterium* metabolic pathway, fructose-6-phosphate phosphoketolase (F6PPK), in 1967, gave a clear distinction of the species and finally differentiated it from others in the Bergey's Manual of 1974 (Ballongue, 1998). Bifidobacteria have a typical glucose metabolism using F6PPK, transaldolase and transketolase, producing mainly L+lactic acid

and acetic acid (Ballongue, 1998; Fandi *et al.*, 2001; Hartemink *et al.*, 1996; Wolin *et al.*, 1998).

Bifidobacteria are gram-positive, anaerobic bacteria (Hartemink *et al.*, 1996) that are commonly found in the colon of mammals. Of the 34 known species 13 are isolated from humans (Leahy *et al.*, 2005). 3-6% of the human colon microflora and 25% of cultivable human colon microflora consist of *Bifidobacterium* species (Hopkins *et al.*, 1998; Leahy *et al.*, 2005). Predominant strains in humans are *B. breve* and *B. infantis* in infants and *B. adolescentis* and *B. longum* in adults (Ballongue, 1998; Hopkins *et al.*, 1998). The bacterial morphology varies depending on the culture medium; they are mainly rods, but can be round, branched or pleomorphic (Ballongue, 1998; Hartemink *et al.*, 1996).

Probiotic activities of *Bifidobacterium* species were first demonstrated in 1958 (Ballongue, 1998). Since then, a number of probiotic mechanisms have been proposed and thus they are commercially used as probiotics e.g. in dairy products (Perrin *et al.*, 2000). Only one strain, *B. dentium*, has been found pathogenic (Ballongue, 1998). Bifidobacteria as probiotics affect the eco-physiology of the large intestinal microbiota and they also interact directly with the host metabolism. The bacteria are able to adhere to the colon and may influence the composition and function of the mucosa. This colonization of the adhesive sites prevents the colonization of pathogens and thus increases host resistance to colon infections (Ballongue, 1998, Desjardins *et al.*, 1990, Hartemink *et al.*, 1996). Nutritional effects caused by bifidobacteria include the production of vitamins and lactic acid (Ballongue, 1998; Leahy *et al.*, 2005). Effects on lactose intolerance, hypercholesterolemia, use of nitrates and bile acids are considered metabolic effects of bifidobacteria in the colon (Ballongue, 1998; Hartemink *et al.*, 1996; Leahy *et al.*, 2005). Bifidobacteria are also shown to have anticarcinogenic and antimutagenic activity (Hartemink *et al.*, 1996; Leder *et al.*, 1999).

1.3.1 *B. longum*

B. longum was first found in 1963 (Reuter, 1963). It is present in the colon of all human age groups (Ballongue, 1998). Its closest relative is *B. infantis*. The distinction of different

Bifidobacterium species can be made by their ability to ferment different sugars. *B. longum* is the only *Bifidobacterium* strain capable of fermenting melezitose and arabinose (Ballongue, 1998). The strain is fairly easy to cultivate, and it has been used a lot in clinical testing. Actually most of the health effect studies have been made on *B. longum* (Ballongue, 1998) and *B. animalis* (Picard *et al.*, 2005).

B. longum has been found to stimulate the immune system in various studies (Picard *et al.*, 2005). It is able to secrete proteins that inhibit the adhesion of potential pathogens (Lievin *et al.*, 2000) and even give the host organism resistance to lethal bacteria, such as *E. coli* O157 (Ishibashi and Yamazaki, 2001) and *Salmonella typhimurium* (Leahy *et al.*, 2005). *B. longum* was found to inhibit carcinogen damage to DNA (Leahy *et al.*, 2005) and colon cancer induction in rats (Gallaher and Khil, 1999; Picard *et al.*, 2005). It has also been found effective against liver tumors (Ballongue, 1998), aberrant crypt foci (Leahy *et al.*, 2005) and antibiotic associated diarrhea (Leahy *et al.*, 2005; Picard *et al.*, 2005).

1.3.2 Freeze-drying

Freeze-drying is a popular, gentle method for the formulation of probiotic bacteria preparations. In freeze-drying, water is removed from a solid surface by direct transfer from solid to gaseous state, when the vapor pressure and surface temperature are below the triple point (Karel, 1975). This drying method has a totally different effect on the dried material than other drying methods, as no liquid phase is present in the process. Mass transfer occurs when the water molecules travel from the material to the condensing chamber (temperature below -50^{o}C). Heat transfer is required to keep the material temperature high enough for this mass transfer to occur at sufficient rate.

The effects of storage time and temperature, as well as different additives on product activity have been studied (Champagne *et al.*, 1996; Foschino *et al.*, 1995). It has been found that sugars, such as lactose and sucrose, are effective cryoprotectants as well as some larger molecules, such as gelatin (Champagne *et al.*, 1996; Mattila-Sandholm *et al.*, 2002). Lactobacilli have shown good survival ratios during freeze-drying: up to 80% viabilities have been obtained (Palmfeldt and Hahn-Hägerdal, 2000). Bifidobacteria, on the other

hand, have been rather sensitive to freeze-drying with survival ratios of around 10% (Maitrot *et al.*, 1997). The use of cryoprotectants should be considered relative to the purpose of the product, e.g. lactose should be avoided when the product is used as a supplement in food for the lactose intolerant. Heat programming (temperature gradients during the drying phase) has been found efficient for the drying of food products, mainly in reducing drying times (Lombraña and Diaz, 1987).

1.3.3 Experiment designs with *Bifidobacterium* species

First pH controlled fermentations and kinetic models of bifidobacteria were reported in 1989 (Desjardins *et al.*, 1990). Studies on cultivation optimization have been such that a single factor is optimized at a time (Mahalakshmi and Murthy, 2000). Temperature and pH effects on *B. bifidum* growth were studied in whey-based media. Galacto-oligosaccharide production with *B. infantis* was optimized using a CCC design for 4 variables: time, temperature, cell and lactose concentrations (Roy *et al.*, 2002).

1.4 Streptomycetes

Streptomycetes are aerobic, pigmented bacteria that form mycelia during submerged cultivations (Figure 3). They are known producers of industrial enzymes and medically important compounds, e.g. antibiotics, polyketides, tetracyclines and antitumor agents. The amount of known products has grown dramatically over the decades, and the market in 2001 was over $30 billion (Demain, 2002). The first antibiotic produced by a streptomycete, streptothricin, was found in 1942 (Watve *et al.*, 2001). In 1995, 55% of the 12 000 secondary metabolites with antibiotic activity were produced by streptomycetes (Weber *et al.*, 2003). By the year 2000, around 3000 antibiotics produced by streptomycetes had been found, and no end to the quest could be seen (Watve *et al.*, 2001). The most common commercial products are streptomycin, tetracycline, erythromycin, nystatin and chloramphenicol. Anthracycline antitumor agents with worldwide clinical use are daunomycin, doxorubicin, idarubicin, epirubicin, pirarubicin, zorubicin and aclacinomycin A. They inhibit the proliferation of cancerous cells by affecting the control

of cell division. They are widely used in the treatments of leukemia, tumors (especially breast and ovary) and HIV induced Kaposi's sarcoma.

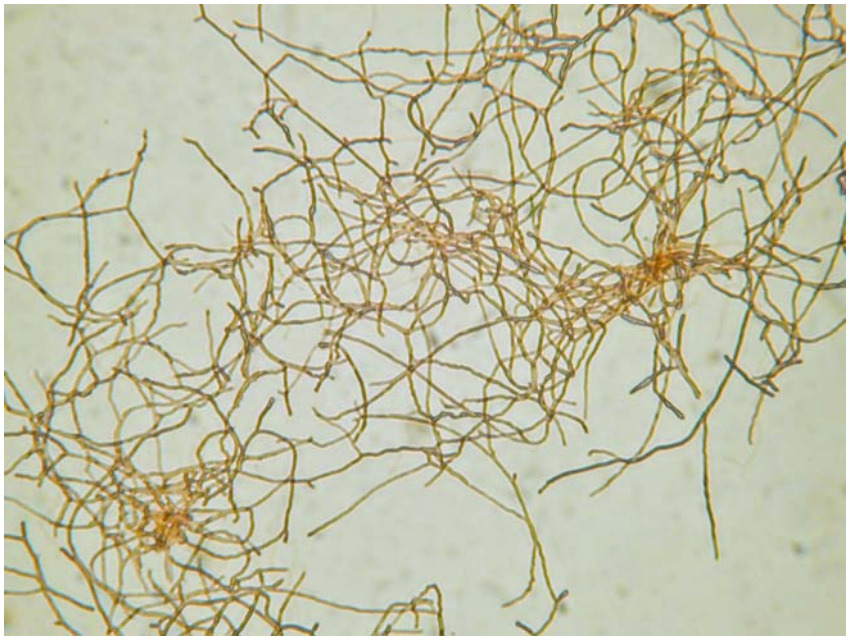

Figure 3. A microscopic image of *Streptomyces peucetius* var. *caesius* N47. This is a Gram-stained slide of a fully grown inoculum, magnification 100 x.

1.4.1 *Streptomyces peucetius*

Streptomyces peucetius is a streptomycete that was isolated from a soil sample in 1957 (Grein, 1987). The species is capable of producing the anthracycline antibiotics aclacinomycin, carminomycin, daunorubicin and doxorubicin. However, it produces a higher proportion of daunorubicin than other daunorubicin producing streptomycetes (Huk and Blumauerán, 1989). Young colonies are typically yellow-pink, turning later to yellow-red and to red-brown as the colonies grow older (Arcamone *et al.*, 1969). The aerial mass can be white or red, and the colors of different colonies can vary significantly (Arcamone *et al.*, 1969). The color depends on the medium and the product, the intermediate ε-rhodomycinone gives a dark red-violet color to cultivations (Arcamone *et al.*, 1969; Huk and Blumauerán, 1989). A mixture of daunorubicin and doxorubicin is currently produced for medical use by Swedish Orphan.

The anthracycline antibiotic biosynthesis route of *S. peucetius* has largely been studied from the precursors malonyl-CoA and propionyl-CoA (Bao *et al.*, 1999; Paulick *et al.*, 1976), to aklanonic acid, aklaviketone, ε-rhodomycinone, carminomycin and daunorubicin (Dickens *et al.*,1995; Lomovskaya *et al.*, 1998; Lomovskaya *et al.*, 1999; Madduri and Hutchinson, 1995b; Stutzman-Engwall and Hutchinson, 1989). The pathway is presented in Figure 4. *Streptomyces* antibiotic regulatory proteins have also been identified and studied (Madduri and Hutchinson, 1995a; Sheldon *et al.*, 2002; Wietzorrek and Bibb, 1997). The most important regulator proteins DnrO, DnrN and DnrI, located in two gene locuses, $dnrR_1$ and $dnrR_2$, are inducers of the antibiotic biosynthesis proteins.

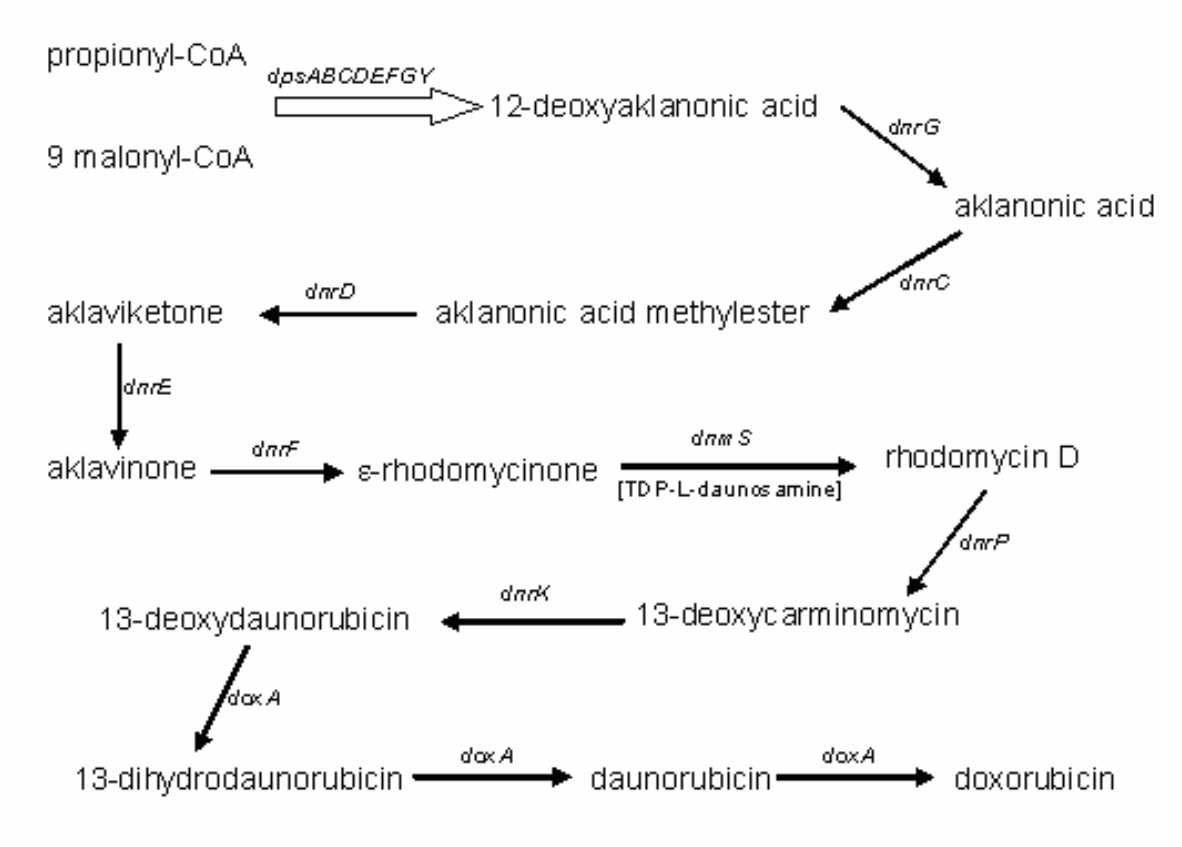

Figure 4. Biosynthesis route of anthracycline antibiotics dauno- and doxorubicin by *S. peucetius*.

1.4.2 *S. peucetius* var. *caesius*

S. peucetius var. *caesius* is a producer of doxorubicin (Arcamone *et al.*, 1969), which is used as an antitumor agent. The strain, like other *S. peucetius* variants (Huk and

Blumauerán, 1989), was originally formed by chemical mutation from the parent strain (Arcamone *et al.*, 1969), which led to the enhanced production of a certain anthracycline compound, in this case, doxorubicin. The strain is capable of producing doxorubicin from different carbon sources including mannose, cellobiose, lactose, fructose, maltose and starch (Guzmán *et al.*, 2005). The biosynthesis is quite similar to the daunorubicin biosynthesis (Figure 4), only proceeding a little further. The medicinal properties of doxorubicin are similar to those of daunorubicin, only generally more effective (Lown, 1993). Three major marketers of doxorubicin in Finland are Pfizer, Schering-Plough and Leiras.

1.4.3 Experiment designs with *Streptomyces* species

Central composite designs have been successfully used with other actinomycetes and other products. Glutamic acid and phosphate concentrations were optimized using a CCC experiment design for the production of a hybrid antibiotic with *S. lividans* TK21 (Sarra *et al.*, 1993). Transglutaminase production with *Streptoverticillium cinnamoneum* was optimized with respect to casein and glycerol concentrations in the production medium using a CCC experiment design (Junqua *et al.*, 1997). The effects of pH and temperature on cellulase-free xylanase production of *Streptomyces* sp. Ab106 were studied using a CCB design (Techapun *et al.*, 2002a). Aeration and agitation rates were optimized for the same using a CCF design (Techapun *et al.*, 2003). The use of mixture designs in actinomycete process optimizations has been less popular. The effects of five different nutrient components on the cellulase-free xylanase production were studied using a mixture design (Techapun *et al.*, 2002b). Growth effects were not evaluated in this study.

1.4.4 MFA of streptomycetes

MFA has been made with different streptomycetes: *S. clavuligerus* (Kirk *et al.*, 2000), *S. coelicolor* (Kim *et al.*, 2004), *S. lividans* (Avignone Rossa *et al.*, 2002), *S. noursei* (Jonsbu *et al.*, 2001) and *S. tenebrarius* (Borodina *et al.*, 2005a). These studies were made using either continuous (Avignone Rossa *et al.*, 2002; Kirk *et al.*, 2000) or batch (Borodina *et al.*, 2005a; Jonsbu *et al.*, 2001; Kim *et al.*, 2004) cultivation data. The scope was mostly to

estimate fluxes through the central carbon metabolism pathways: EMP, ED, PPP, the shikimate pathway, TCA and anaplerotic reactions. The results are reviewed in Table 1.

Table 1. MFA results from studies on different streptomycetes.

Organism	Observed pathway effects when shifting from growth to production	Reference
S. clavuligerus	PPP ↑ urea cycle ↑	Kirk *et al.*, 2000
S. coelicolor	PPP ↑ EMP ↑ TCA ↓ shikimate pathway ↑	Kim *et al.*, 2004
S. lividans	PPP ↓ EMP ↑	Avignone Rossa *et al.*, 2002
S. noursei	PPP ↓ TCA ↑	Jonsbu *et al.*, 2001
S. tenebrarius	ED ↓ PPP ↑ EMP ↑	Borodina *et al.*, 2005a

1.4.5 Modeling of *Streptomyces* production processes

The attempt to model *Streptomyces* production processes began in the 1970's. Various strategies have been used in this complex area ranging from simple kinetic approaches to compartment models, population models and hybrid models. A modified Monod model, with both carbon and nitrogen form of the equation, and an expression for cell death, was used on *S. erythreus* cultivation and erythromycin production (Ettler and Votruba, 1980). Monod kinetics was also used in the simulation of biomass, DNA, RNA, proteins and glucose with different *Streptomyces* species (King, 1997). The model was developed using *S. tendae*, and further expanded by process phase time constants to other streptomycetes (King and Büdenbender, 1997). Another study used the logistic approach in biomass estimation and Luedeking-Piret-type equations for everything else (Elibol and Mavituna, 1999). A fed-batch process for aceto-isovaleryl tylosin production by *S. thermotolerans* was modeled using a substrate and product inhibition model inconsistent with the typical growth kinetics (Huang *et al.*, 2001). Compartment modeling was applied to nourseothricin production by *S. noursei* for the purpose of optimizing the fed-batch production process (Peissker *et al.*, 1984). In addition to these, some models have been published in Chinese in the recent years.

2 Aims of the study

B. longum and *S. peucetius* are two totally different bacterial species with respect to environmental conditions and process objective. These were used as model organisms in this study. The objective of the *B. longum* process was to produce viable biomass suitable for use as food additive. The objective of the *S. peucetius* process was to produce a medically significant compound toxic to the organism.

The aims of the study were:

- to find suitable complex medium components for the cultivation of *S. peucetius*,
- to determine critical parameters for the cultivation of *B. longum*,
- to optimize the critical cultivation parameters concerning *B. longum* growth,
- to optimize environmental conditions for an efficient ε-rhodomycinone production process with *S. peucetius*,
- to find a strategy for the efficient freeze-drying of *B. longum*,
- to obtain information on the steady state chemostat characteristics of *S. peucetius*,
- to test the effects of environmental changes on ε-rhodomycinone production using a steady state continuous cultivation of *S. peucetius*,
- to analyze intracellular fluxes from the continuous cultivation experiments and
- to construct a predictive batch process model for *S. peucetius* using kinetic equations and neural networks

3 Materials and methods

3.1 Bacterial strains

Bifidobacterium longum ATCC 15707 and *Streptomyces peucetius* var. *caesius* N47 were used as model organisms. Both were stored as frozen working stocks at -80°C. *B. longum* inoculums were prepared in 15 ml test tubes containing MRS supplemented with L-cysteine-HCl·H_2O (1 g l^{-1}, MRSC) at 37°C. *S. peucetius* inoculums were prepared in 250 ml shake flasks at 30°C and 330 rpm. The 50 ml inoculum medium contained in g l^{-1} glucose 20, starch 20, soy peptone or Pharmamedia 5, yeast extract 2.5, NaCl 3, $CaCO_3$ 3, KH_2PO_4 1 and $MgSO_4$ 0.49, as well as the following trace elements in mg l^{-1}: $FeSO_4$ 1.1, $MnCl_2$ 2.2, $CuCl_2$ 0.8 and $ZnSO_4$ 3.9.

3.2 Bioreactor cultivations

Reactor types used were Biostat Q (1.0 l) and Biostat MD (2.0 l) (B. Braun Biotech International, Germany) with MFCS-program (versions 1.1 and 2.1, B. Braun) to monitor the cultivations. Off-gas analysis was made by VG Prima 600 (VG Gas Analysis Systems Ltd., Middlewich, Cheshire, UK) and Omnistar GSD 301 O gas analysis system (Pfeiffer Vacuum, Assler, Germany).

3.3 Freeze-drying

A Christ Alpha 2-4 laboratory scale freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) was used with a Polystat cc3 programmable bath (Peter Huber Kältemaschinenbau GmbH, Germany). Temperature controlled experiments were made to determine the effects of drying time and temperature on freeze-drying. Temperature programming experiments were conducted in order to reduce the drying time and improve

the product activity. Frozen 1 ml aliquots from an overnight batch cultivation were used in the experiments.

3.4 Analytical methods

3.4.1 Cell mass analysis

B. longum cell mass was evaluated with optical density (OD) measurements. *S. peucetius* cell mass was analyzed by filtration of 1-5 ml samples with a pre-incubated 0.2 µm hydrophilic polypropylene membrane. The maximum specific growth rate (μ_{max}) was determined from the slope of the natural logarithms of 3 successive measurements at the steepest growth phase.

3.4.2 Cell viability analysis

S. peucetius cell viabilities were analyzed from diluted samples as colony forming units (cfu) on nutrient agar plates incubated at 30°C for 7 days. μ_{max} was determined from the logarithmic values. The freeze-dried *B. longum* product was weighed and resuspended in 10 ml saline. The viability was determined anaerobically on MRSC agar plates at 37°C.

3.4.3 Glucose analysis

Glucose was analyzed from cell free samples by YSI 2700 Select (Yellow Springs Instrument Inc., U.S.A.). The maximum glucose consumption rate (r_G) was calculated using the measured concentrations and sampling times. The relative glucose consumption time (t_G) was calculated from the measured initial glucose concentration and the end time of base consumption during the cultivation.

3.4.4 ε-rhodomycinone

Samples for ε-rhodomycinone analysis from *S. peucetius* cultivations were analyzed with two different methods. The first method comprised extraction with dichloromethane (DCM). 500 µl sample was thoroughly mixed with 200 µl phosphate buffer (pH 7) and 200

µl methanol. ε-rhodomycinone was extracted twice from this solution by addition, mixing and separation of 500 µl DCM. The DCM was evaporated from the samples at room temperature. The second method was developed for the accurate analysis of total ε-rhodomycinone produced. 1 ml sample was treated with 200 µl 1.2 M borate-phosphoric acid buffer (pH 2.0) and centrifuged in a glass test tube. The product was extracted from the cells by treating the precipitate with methanol. The methanol was evaporated from the samples at room temperature.

3.4.5 HPLC

Dry ε-rhodomycinone samples were dissolved in 1 ml 3.8% trifluoroacetic acid (TFA) and 24% acetonitrile (ACN) and analyzed with high performance liquid chromatography (HPLC, Waters, Milford, MA, USA). An XTerra RP_{18} column (Waters) was used at 30°C with a UV detector (λ = 254 nm). A 16 min linear elution gradient was applied to the eluents 0.05% TFA (from 76 to 1%) and ACN (from 24 to 99%).

Acetate, α-ketoglutarate, citrate, ethanol, formate, fumarate, lactate, pyruvate and succinate were analyzed from culture supernatants using an Aminex HPX-87H column (Bio-Rad Laboratories, USA) at 65°C with 5 mM H_2SO_4 as the mobile phase. A pre-column of the same type was included in the system.

3.5 Experiment designs

Different experiment designs were used for different objectives. With *B. longum* a reasonably simple and cost-effective medium was already known, but no information could be found on component level significances or optimal amounts. A suitable starting ground was thus a fractional factorial estimation of the most significant variables having an effect on growth. With *S. peucetius* nothing was known at the beginning, so the process needed to start with medium development and proceed with the optimization of environmental conditions. The freeze-drying of *B. longum* was a complex process with little room for experiment design variables. Thus the two objects for optimization were time and

temperature with response surface methods. All experiment designs were made and evaluated using Modde 4.0 (Umetri, Umeå, Sweden).

3.5.1 Mixture design

A mixture design was constructed in order to investigate the effects of 10 complex medium components on the cultivation of *S. peucetius*. The components containing no particulate matter were bacto peptone, beef extract, cotton seed extract, Pharmamedia extract, malt extract, nutrient broth, soy peptone, tryptone, yeast extract and YM broth. The experiment design is shown in Table 2. The cultivations were performed in Bioscreen C (Labsystems, Helsinki, Finland) using a cultivation volume of 400 µl. All experiments contained a total of 10 g l^{-1} complex medium components. The Bioscreen C analyzer measured the cultivation optical densities (OD, λ = 600 nm) at 30 min intervals. Lag-phase length (Lag), μ_{max} and absorbance change during growth phase (dExp) were determined and calculated from the Bioscreen results, and used as model responses.

3.5.2 Fractional factorial design

The estimation of critical parameters in the cultivation of *B. longum* was conducted using a fractional factorial design. Typical cultivation medium components used with lactic acid bacteria were chosen as factors (yeast extract, glucose, Tween 80, $MgSO_4$ and phosphates) as well as pH and propionic acid. pH is a common environmental factor and to our knowledge its significance in the neutral zone on bifidobacterial cultivations has not been reported. Propionic acid has been proposed to stimulate the growth of bifidobacteria (Hartemink *et al.*, 1996). A 2^{8-4} fractional factorial design was constructed in order to evaluate the significance of pH, and the amounts of yeast extract, glucose, L-cysteine-$HCl \cdot H_2O$, Tween 80, propionic acid, $MgSO_4$ and phosphates in the medium, in *B. longum* cultivations. The experiment design is presented in Table 2. The cultivation volume was 400 ml and the temperature was controlled at 37°C. μ_{max} and t_G were used as responses.

3.5.3 Full factorial design

A two level full factorial screening design was constructed for the investigation of temperature programming in the freeze-drying of *B. longum*. The experiment design is shown in Table 2. The log(a) values obtained in the activity analysis of the drying product were used as responses.

3.5.4 CCF

A CCF experiment design was constructed for the investigation of temperature, pH and DO on cell growth, glucose consumption and ε-rhodomycinone formation during the growth phase of *S. peucetius*. The design is shown in Table 2. Another CCF design was constructed for the investigation of temperature, pH and stirring rate on ε-rhodomycinone production after the growth phase. This design is also shown in Table 2. A temperature transformation to K^{-1} was used in both models to obtain linearity concerning temperature effects.

The growth phase experiments were conducted in Biostat MD reactors with 1.0 l working volume. After a 40-80 h growth phase the medium was transferred to two production phase reactors. The production phase experiments were made in two parallel Biostat Q reactors with 400 ml working volumes, one with and another without aeration. Every production phase was continued for 88 h. Samples were taken every 8 hours from both growth and production phase. μ_{max}, $\ln(r_G)$ and $\ln(r_e)$ were used as responses in the growth phase study. The maximum ε-rhodomycinone formation rate was calculated from the aerated (p_+) and non-aerated (p'_-) production batch and used as production model response.

The effects of time (t) and temperature (T) on the freeze-drying of *B. longum* were studied using a CCF design. The center point was analyzed only once. The experiment design is shown in Table 2. Again, log(a) was used as the model response.

Table 2. Experiment designs used in the study.

Exp.	*S. peucetius* mixture design [a]										*B. longum* fractional factorial design [b]							
	SP	TR	CE	YE	NB	BE	ME	BP	YM	FM	pH	YE ($g\ l^{-1}$)	T80 ($g\ l^{-1}$)	Cy ($g\ l^{-1}$)	Glu ($g\ l^{-1}$)	Mg ($g\ l^{-1}$)	PA (mM)	PO4 ($g\ l^{-1}$)
1	1	0	0	0	0	0	0	0	0	0	6.2	15	0.5	0.5	10	0.2	0	4
2	0	1	0	0	0	0	0	0	0	0	7.0	15	0.5	0.5	10	0.5	25	8
3	0	0	1	0	0	0	0	0	0	0	6.2	30	0.5	0.5	15	0.2	25	8
4	0	0	0	1	0	0	0	0	0	0	7.0	30	0.5	0.5	15	0.5	0	4
5	0	0	0	0	1	0	0	0	0	0	6.2	15	1	0.5	15	0.5	25	4
6	0	0	0	0	0	1	0	0	0	0	7.0	15	1	0.5	15	0.2	0	8
7	0	0	0	0	0	0	1	0	0	0	6.2	30	1	0.5	10	0.5	0	8
8	0	0	0	0	0	0	0	1	0	0	7.0	30	1	0.5	10	0.2	25	4
9	0	0	0	0	0	0	0	0	1	0	6.2	15	0.5	1	15	0.5	0	8
10	0	0	0	0	0	0	0	0	0	1	7.0	15	0.5	1	15	0.2	25	4
11	0.14	0	0.14	0.14	0.02	0.14	0.14	0.14	0	0.14	6.2	30	0.5	1	10	0.5	25	4
12	0.14	0.14	0	0.14	0.02	0.14	0.14	0.14	0	0.14	7.0	30	0.5	1	10	0.2	0	8
13	0.14	0.14	0.14	0	0	0.14	0.14	0.14	0.02	0.14	6.2	15	1	1	10	0.2	25	8
14	0.14	0.14	0.14	0.14	0.02	0.14	0.14	0	0	0.14	7.0	15	1	1	10	0.5	0	4
15	0	0.14	0.14	0.14	0	0.14	0.14	0.14	0.02	0.14	6.2	30	1	1	15	0.2	0	4
16	0.14	0.14	0.14	0.14	0	0	0.14	0.14	0.02	0.14	7.0	30	1	1	15	0.5	25	8
17	0.14	0.14	0.14	0.14	0	0.14	0	0.14	0.02	0.14								
18	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1								
19	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1								
20	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1								

	B. longum full factorial design [c]			*B. longum* CCC design [d]			*S. peucetius* CCF design [e]			*S. peucetius* CCF design [f]			*B. longum* CCF design [g]	
	t_1 (h)	t_2 (h)	t_3 (h)	T (^oC)	C_G ($g\ l^{-1}$)	C_Y ($g\ l^{-1}$)	pH	T (^oC)	DO (%)	pH	T (^oC)	stir (rpm)	t (h)	T (^oC)
1	2	2	4	36	10	20	6	25	0	6	25	100	20	-10
2	10	2	4	36	10	40	8	25	0	8	25	100	60	-10
3	2	10	4	36	20	20	6	35	0	6	35	100	20	25
4	10	10	4	36	20	40	8	35	0	8	35	100	60	25
5	2	2	12	40	10	20	6	25	30	6	25	400	20	0
6	10	2	12	40	10	40	8	25	30	8	25	400	60	0
7	2	10	12	40	20	20	6	35	30	6	35	400	40	-10
8	10	10	12	40	20	40	8	35	30	8	35	400	40	25
9	10	6	8	38	15	15	6	30	15	6	30	250	40	0
10	6	10	8	38	15	45	8	30	15	8	30	250		
11	6	6	4	38	7.5	30	7	25	15	8	25	250		
12	6	6	8	38	22.5	30	7	35	15	7	35	250		
13	6	6	8	35	15	30	7	30	0	7	30	100		
14	6	6	8	41	15	30	7	30	0	7	30	400		
15				38	15	30	7	30	15	7	30	250		
16				38	15	30	7	30	15	7	30	250		
17				38	15	30	7	30	15	7	30	250		

a) Mixture design for complex medium optimization of *S. peucetius* cultivation. The factors are soy peptone (SP), tryptone (TR), corn extract (CE), yeast extract (YE), nutrient broth (NB), beef extract (BE), malt extract (ME), bacto peptone (BP), YM medium (YM) and Pharmamedia extract (FM). b) Fractional factorial design (2^{8-4}) for the evaluation of critical parameters of the *B. longum* cultivation. The factors are pH, yeast extract (YE), Tween 80 (T80), L-cysteine-HCl monohydrate (Cy), glucose (Glu), $MgSO_4$ (Mg), propionic acid (PA) and phosphates (PO4). c) Full factorial experiment design for a temperature program experiment in the freeze-drying of *B. longum*. The factors are time for temperature raise from -10 to 0^oC (t_1), time for temperature raise from 0 to $+10^oC$ (t_2) and temperature hold time at $+10^oC$ (t_3). d) CCC design for *B. longum* growth optimization of temperature (T) and concentrations of glucose (C_G) and yeast extract (C_Y). e and f) CCF designs for the optimization of *S. peucetius* growth and production phase. The growth phase design factors are pH, temperature (T) and dissolved oxygen (DO). The production phase design factors are pH, temperature (T) and stirring rate (stir).
g) CCF design for the preliminary freeze-drying experiment of *B. longum*. The design factors are time (t) and temperature (T).

3.5.5 CCC

After the estimation of the critical parameters of the *B. longum* cultivation, a further optimization of T, yeast extract (C_Y) and glucose (C_G) concentration was carried out. A CCC design with a star distance of 1.5 (Table 2) was used. The cultivation volume in all experiments was 500 ml. μ_{max}, $\ln(r_G)$ and t_G were used as responses.

3.6 Modeling

Models were obtained from the literature and compiled in formats compatible with Matlab 6.0 software (MathWorks, Natick, MA, USA). Some modeling techniques were combined, especially in the kinetic modeling section, to yield a thorough process model capable of realistic simulation of the experimental batch results.

3.6.1 MFA

The metabolic model for the MFA of *S. peucetius* was modified from Borodina *et al.* (2005b). The model was a genome based metabolic network constructed for *S. coelicolor* A3(2). The authors proposed that the model can be used as a general model of *Streptomyces* metabolism. Rare and complex sugars as well as the secondary metabolites were removed from the model, and the following reactions for ε-rhodomycinone production were added.

Polyketide synthase: propionyl-CoA + acp + 9 malonyl-CoA → 9 CO_2 + 10 CoA + NHACP

Ketoreductase: NHACP + NADH → NAD + NHCACP

Aromatase: NHCACP → NHAACP

Cyclase: NHAACP + 2 NADH → 2 NAD + 12-deoxyaklanonic acid + acp

Oxygenase: 12-deoxyaklanonic acid + O_2 → aklanonic acid

Methylase: aklanonic acid + S-adenosyl-L-methionine → S-adenosyl-L-homocysteine + H + aklanonic acid methyl ester

Cyclase: aklanonic acid methyl ester → aklaviketone

Ketoreductase: aklaviketone + NADH → NAD + aklavinone

Hydroxylase: aklavinone → H + ε-rhodomycinone

NHACP, NHCACP and NHAACP are acyl carrier protein (acp) bound intermediates of 12-deoxyaklanonic acid synthesis.

The network contained 77 external and 438 internal metabolites, and 624 reactions, 191 of them being reversible. Matlab 6.0 was used in the calculations with the aid of the software package FluxAnalyzer 5.2 (Max Planck Institute, Magdeburg, Germany; Klamt *et al.*, 2002). HPLC, biomass and gas analysis data were used in building the constraints. As the network was underdetermined, flux optimization was used in the network analysis. Rates of EMP, ED, PPP, TCA and the shikimate pathway were evaluated under different conditions.

3.6.2 Kinetic models

Different kinetic equations were fitted to the cultivation data and simulated using Matlab 6.0 software and the software package Simulink 4.0. The equations used for growth estimation were Monod (Eq. 1), Teissier (Eq. 2) and Contois (Eq. 3) kinetics as well as the logistic equation (Eq. 4). For glucose consumption, a Luedeking-Piret type equation was used (Eq. 5)

$$\mu = \mu_{\max} \frac{S}{S + K_M} \qquad (1)$$

$$\mu = \mu_{\max} \left(1 - e^{\frac{S}{k}} \right) \qquad (2)$$

$$\mu = \mu_{\max} \frac{S}{S + K_C X} \qquad (3)$$

$$\mu = \mu_{\max} \left(1 - \frac{X}{X_{\max}} \right) \qquad (4)$$

$$\frac{dS}{dt} = -X \left(\frac{\mu}{Y_{XS}} + \frac{q_{CO_2}}{Y_{CS}} + m_S \right) \qquad (5)$$

The lag-phase was included in the model with a CO_2 trigger of 0.09 g l^{-1} h^{-1} and a transition term $\mu_{\max} \cdot t$ (Swinnen *et al.*, 2004) as a multiplier to slow down the initial increase of μ.

Kinetics of product formation was introduced into the best modeling system with the following equations (Eq. 6 and Eq. 7).

$$\frac{dX}{dt} = \mu X + \alpha \frac{dP}{dt} \qquad (6)$$

$$\frac{dP}{dt} = V_{\max} X \left(1 - \frac{P}{P_{\max}} \right) \qquad (7)$$

Parameter fittings were done with an optimizing random search protocol developed for this study. The idea of this protocol (modified Monte Carlo method) was to cut down the parameter search space by moving its central point toward the best fit and reducing the size of the space simultaneously. This was done iteratively, and the iteration loops were repeated so that the size of the parameter space was closing the desired parameter accuracy. The initial boundaries for this method can be set according to the known constraints, which is not the case in many optimization algorithms.

3.6.3 Neural network model

The neural network models were done using fully connected feed-forward networks, where every neuron is connected to each neuron in the next forward layer. Input layer neurons perform no computations, but only transmit the normalized values to each connected neuron in the hidden layer. Each input and output node is assigned to measured input and output variables, respectively. The transfer functions used in the hidden and output layer neurons were the logistic sigmoid transfer function and hyperbolic tangent function. The back propagation training algorithm (Rumelhart *et al*., 1986; Werbos, 1974) was used in the study with a neural network program developed in our laboratory (Eerikäinen, 1993).

4 Results and discussion

4.1 *B. longum* process optimization (I)

4.1.1 Critical cultivation parameters

The normalized coefficients for the responses μ_{max} and t_G in the fractional factorial design are shown in Figure 5. R^2 and Q^2 were satisfactory for a fractional factorial design with both responses, i.e. the values were over 0.8 and 0.2, respectively. The P value showed that the model was significant for t_G. The most significant effects on both responses were yeast extract, cysteine and glucose. Yeast extract and glucose had a significant negative effect on t_G and cysteine a positive effect on μ_{max}. pH, Tween 80, $MgSO_4$, propionic acid and phosphate effects were not significant. Yeast extract and glucose were chosen as optimization parameters with temperature.

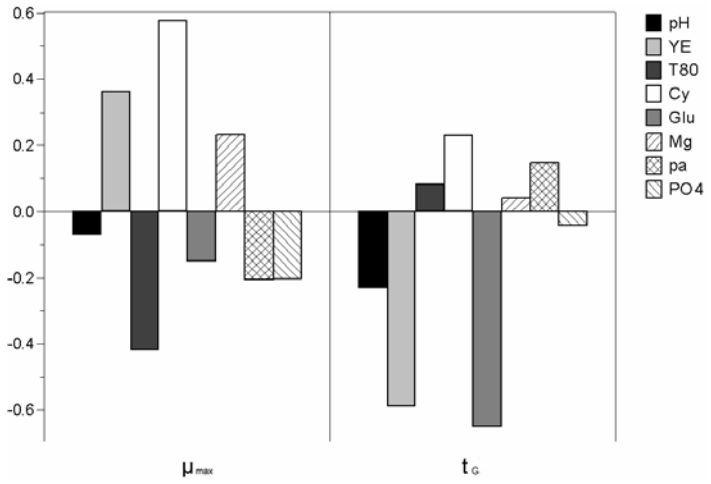

Figure 5. Determination of critical cultivation parameters while growing *B. longum*. The figure shows normalized model coefficients for the variables μ_{max} and t_G.

The critical parameter estimation experiments showed that the most significant factor improving μ_{max} was L-cysteine (Fig. 5). Cysteine is used in the cultivation of oxygen-sensitive organisms as a protecting agent, especially when no nitrogen flushing is applied.

It is, however, an expensive medium component, and its extensive use should thus be carefully considered. Cysteine had no improving effects on t_G and its concentration was not used as an optimization parameter in the CCC experiments. The potential benefits of cysteine to the cultivation depend on the cultivation vessel and agitation type, as these are the most important factors that have an effect on the DO in the medium (Kiviharju *et al.*, 2004).

4.1.2 Optimization of critical parameters

The response surface of μ_{max} in the optimization experiments is shown in Figure 6. The statistic parameters showed that the model was excellent. The most important factor was temperature. The best μ_{max} values were obtained with high yeast extract concentration and temperature values and low glucose concentration values.

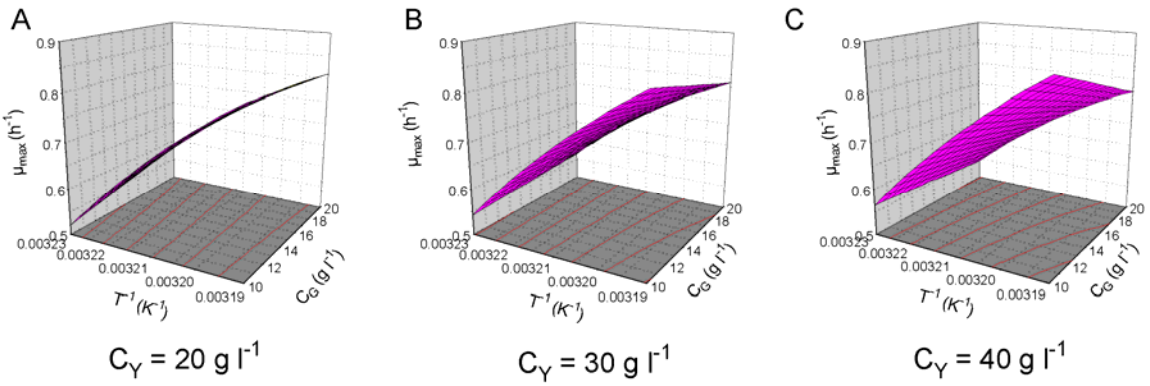

Figure 6. Response surfaces of μ_{max} in the optimization of *B. longum* cultivations at different yeast extract concentrations.

The response surface of $\ln(r_G)$ in the optimization experiments is shown in Figure 7. The statistic coefficients for this model were excellent as well. The most important factor was glucose concentration. The best $\ln(r_G)$ values were obtained with high temperature and glucose concentration values combined with yeast extract concentrations of 30...35 g l^{-1}.

The response surface of t_G in the optimization experiments is shown in Figure 8. The statistic parameters showed that this model, as all others in this experiment design, turned out excellent. The most important factor was glucose concentration. The smallest t_G values

were obtained with high glucose concentration and temperature values and yeast extract concentrations over 32 g l^{-1}.

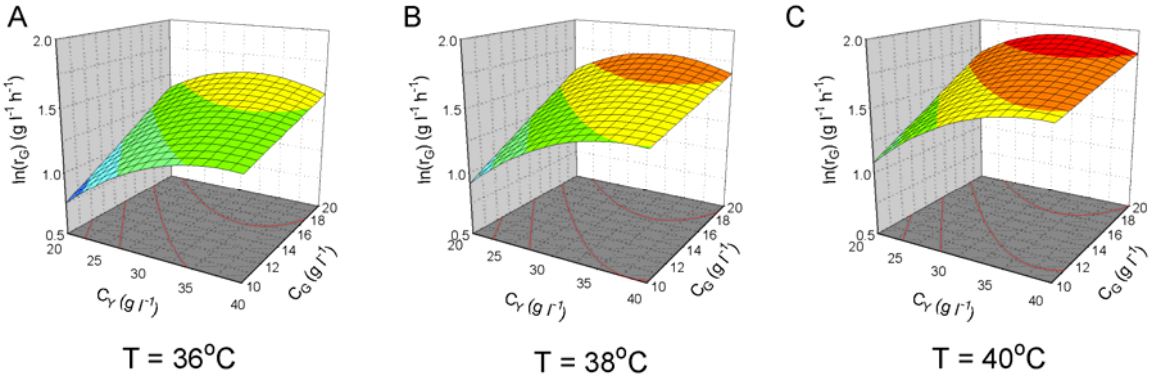

Figure 7. Response surfaces of $\ln(r_G)$ in the optimization of *B. longum* cultivations at different temperatures.

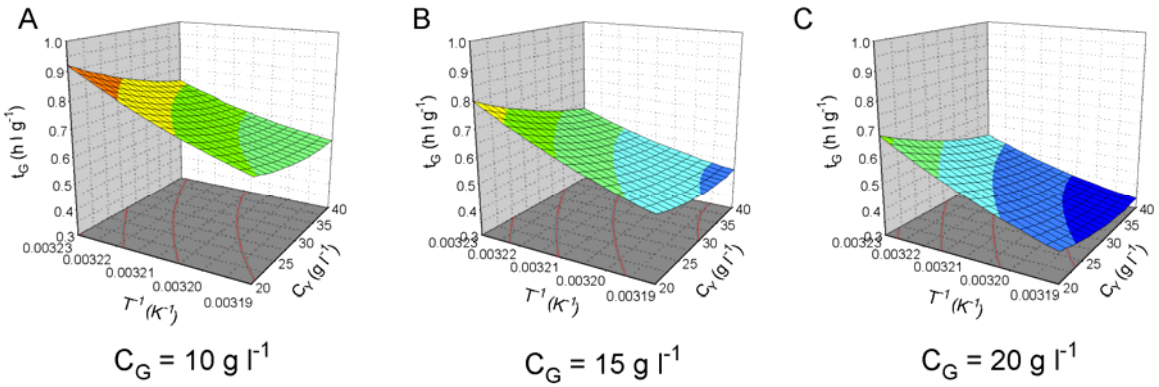

Figure 8. Response surfaces of t_G in the optimization of *B. longum* cultivations at different glucose concentrations.

The CCC optimization experiments showed that the best cultivation results were obtained at 40°C with 35 g l^{-1} yeast extract and 20 g l^{-1} glucose (Figs. 6, 7 and 8). In contrast to an earlier study (Desjardins *et al.*, 1990) implying product inhibition on growth while using substrate concentrations over 10 g l^{-1}, our results showed that a higher substrate concentration yielded better growth. This result did not depend on the amount of complex medium components. A reason for the controversy might be the fact that pH control was applied with a different pH controlling agent, ammonia, which in another study was found to inhibit the growth of *B. longum* (Song *et al.*, 2003).

4.1.3 Freeze-drying

The response surface for log(a) obtained in the constant temperature freeze-drying experiments is shown in Figure 9. The obtained model shows that the product activity increases as the drying time increases and the temperature decreases. This might be due to the partial melting of the product before sufficient evaporation is achieved. The model was found almost significant and the R^2 and Q^2 values satisfactory. The best activity results were obtained with temperatures below 0°C and times over 40 h.

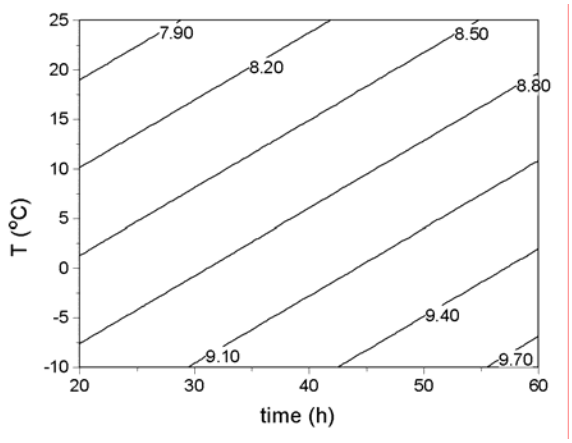

Figure 9. Response surface of log(a) in time and temperature optimization of the freeze-drying of *B. longum*.

The response surfaces of log(a) in the temperature programming experiment are shown in Figure 10. The model was found significant and the R^2 and Q^2 values satisfactory. The most important factor was t_3. The general result was that increasing t_3 and decreasing t_1 increased product activity.

Freeze-drying is a complex process that is affected by the freezing step, apparatus conditions and, as presented in this study, the sample environment conditions. Drying times over 40 h and temperatures below 0°C yielded the best activity results in the constant temperature freeze-drying experiment. When using temperature programs, a 24 h drying time resulted in a higher product activity than the constant temperature experiments. This implies that temperature programming is an effective way to improve the viability of

bacteria in freeze-drying and reduce process time. A 50 h drying time at -10°C would yield a logarithmic product activity of 9.57 with the obtained model for constant temperature freeze-drying. The 24 h temperature program model with 2 h, 10 h and 12 h phases would yield a logarithmic product activity of 9.99. Temperature programming can thus increase product activity by over 160% while decreasing drying time over 50%.

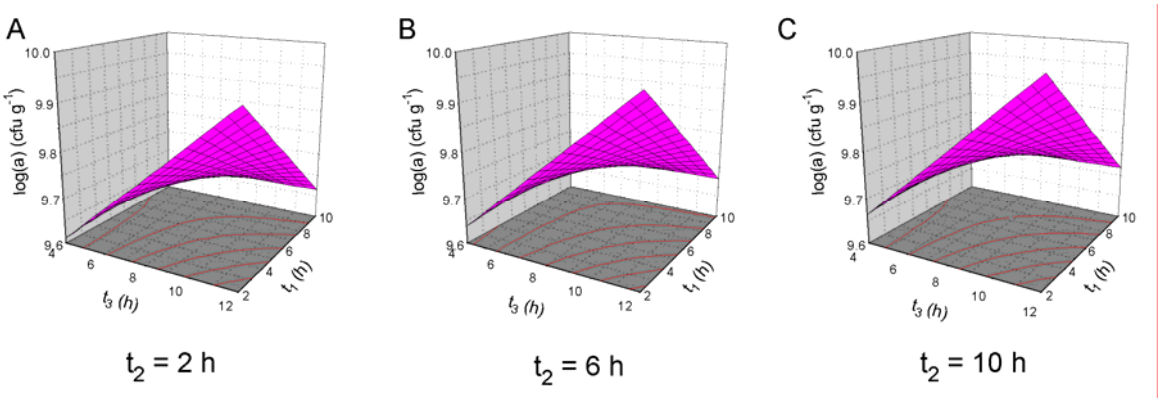

Figure 10. Response surface of log(a) in the temperature programming experiments of *B. longum* freeze-drying with different phase 2 lengths.

4.2 *S. peucetius* var. *caesius* process optimization (II)

4.2.1 Medium component screening

The cultivation responses Lag, μ_{max} and dExp are shown in Figure 11 as normalized effects of the complex medium components. The R^2 and P values were satisfactory for Lag and dExp. The effects lengthening Lag the most were corn extract, malt extract and yeast extract. The biggest stimulating effects concerning the same were found with soy peptone and beef extract. The strongest positive effects on μ_{max} were obtained with soy peptone, bacto peptone and tryptone. Significant negative effects were obtained with corn extract and malt extract. The dExp effects were the best with soy peptone, tryptone, beef extract and bacto peptone. Significant negative effects were obtained with corn extract and malt extract.

A clear complex medium is not always advantageous for the production of anthracyclines with actinomycetes (Macedo *et al.*, 1999). It is, however, a requirement for the efficient

monitoring of cultivations with traditional methods e.g. optical density or cell dry weight measurements as well as some on-line measurement methods. The goal in the mixture design experiments was to rapidly determine the complex medium components for the successful cultivation of the organism. The ε-rhodomycinone production with the components beneficial for growth was not investigated.

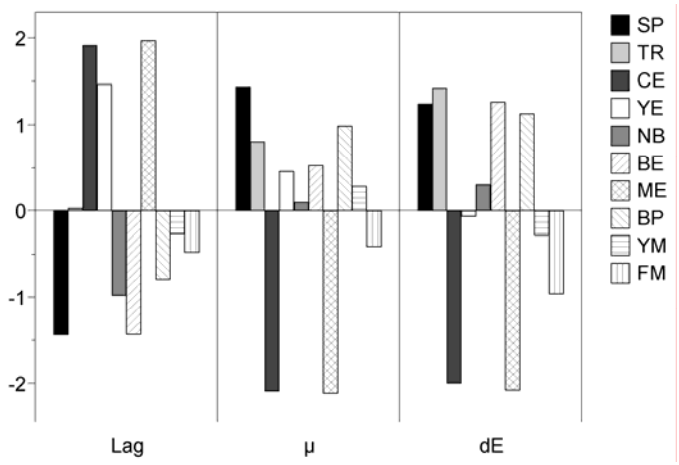

Figure 11. Normalized coefficients in the medium component screening of *S. peucetius* cultivation for the responses Lag, μ_{max} and dExp.

4.2.2 Process optimization

The response surfaces of μ_{max} in the optimization experiments are shown in Figure 12. The statistical parameters showed that the model was almost significant. The most significant interacting factors on μ_{max} were temperature and DO. In all pH values both high temperature and DO values yield the biggest μ_{max} values.

The response surfaces for $\ln(r_G)$ are shown in Figure 13. The statistical parameters suggest that the obtained model was very significant. The most significant interactions on $\ln(r_G)$ were temperature and pH, and temperature and DO. All response surfaces indicate that a high value for all factors yields the biggest $\ln(r_G)$ values.

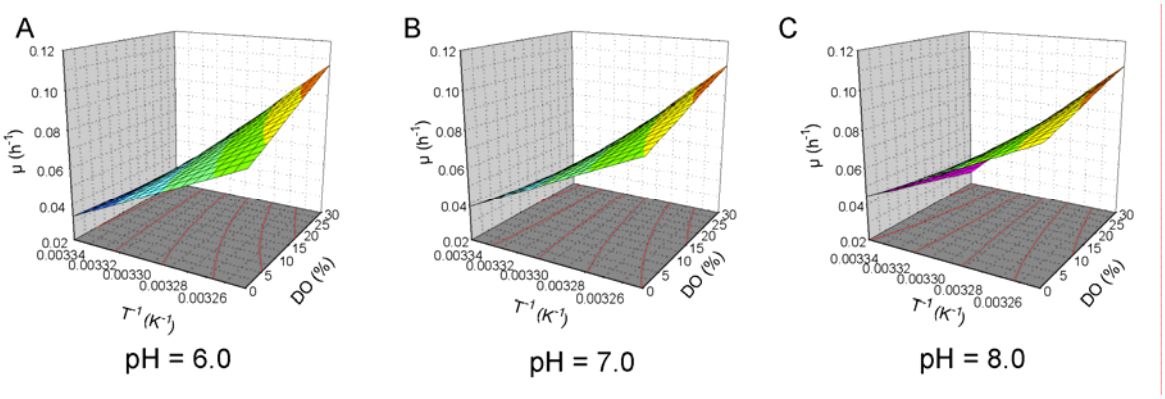

Figure 12. Response surfaces of μ_{max} in the optimization of *S. peucetius* cultivation at different pH values.

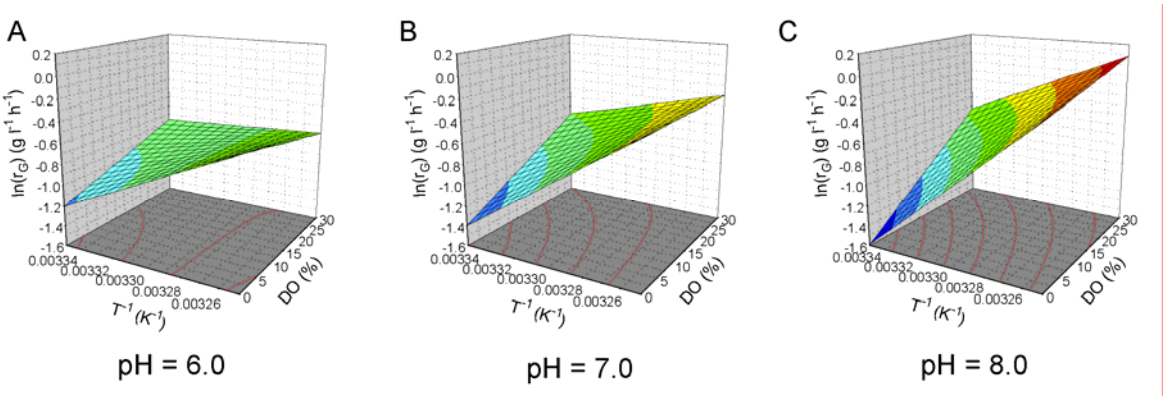

Figure 13. Response surfaces of $\ln(r_G)$ in the optimization of *S. peucetius* cultivation at different pH values.

The response surfaces of $\ln(r_e)$ are shown in Figure 14. The statistical parameters suggest that the model for $\ln(r_e)$ was more significant than the lack of fit. The most significant interacting factors on $\ln(r_e)$ were temperature and pH. The best values were obtained with DO 30% and temperatures around 30°C.

The metabolic activities of cells are clearly temperature dependent. This was shown in the growth phase experiments, as an increase in temperature increased both μ_{max} and $\ln(r_G)$. Glucose repression of anthracycline formation in *S. peucetius* var. *caesius* has been reported (Escalante *et al.*, 1999; Segura *et al.*, 1997) as well as a carbon repression on nystatin production by *S. noursei* (Jonsbu *et al.*, 2002). The cultivation experiments in temperatures below 30°C showed a glucose consumption behavior similar to the reported

repression cultivations (Escalante *et al.*, 1999); glucose consumption in the 25°C cultivations ceased at around 8 g l^{-1}, since in the higher temperature cultivations all glucose was consumed.

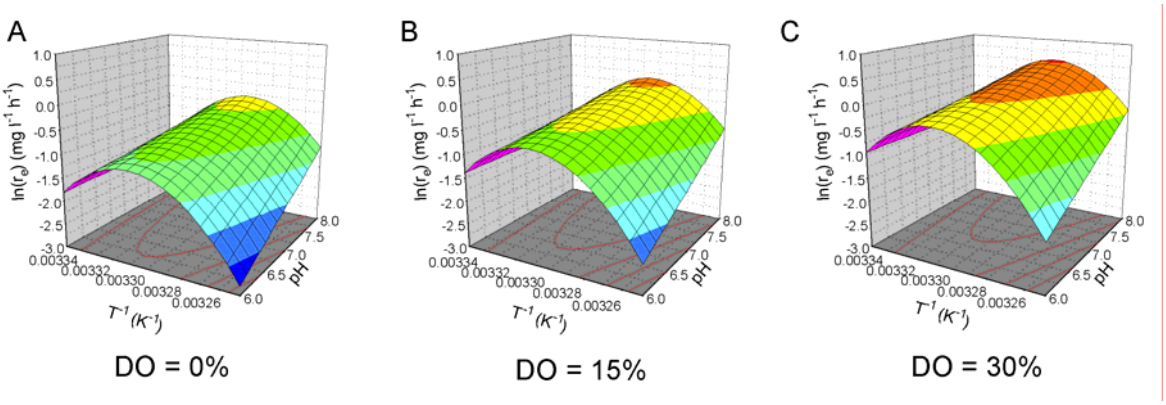

Figure 14. Response surfaces of $\ln(r_e)$ in the optimization of *S. peucetius* cultivation at different DO values.

DO control increased all growth phase responses μ_{max}, $\ln(r_G)$ and $\ln(r_e)$. It has been reported that DO control can have a positive effect on antibiotic production by streptomycetes (Rollins *et al.*, 1988), which is supported by our results in the growth phase experiments (Figure 14); the average production level increased when the DO was increased.

The p_+ showed a product decay rate rather than a product formation rate. The statistical parameters implied that the model was a bad representation of the data. The results were also evaluated using a modified CCF design with pH and temperature changes and stirring rate as design factors. The R^2 and Q^2 values were better for p'., although still not good. The P value, on the other hand, showed that the model for both responses was more significant than its lack of fit. Better productivities were obtained in the non-aerated production phase and the response surface for pH and temperature change with stirring rate 400 rpm is shown in Figure 15. The highest productivities were obtained with the biggest positive changes in pH and decreasing temperatures.

The production phase results were rather complicated to interpret, as the start point differed in medium composition (substrate and metabolite amounts) and viable cell counts. Aerated

production phase conditions seemed to destroy the ε-rhodomycinone faster than non-aerated production conditions. Increasing the stirring rate increases the oxygen transfer to the cultivation medium, and this can increase metabolic activities in the cells aiding product decay. The modified model showed that aeration in the production phase coupled with a pH decrease resulted in rapid ε-rhodomycinone decay in the medium. In non-aerated production phases a pH change resulted in better productivity than in experiments without pH change.

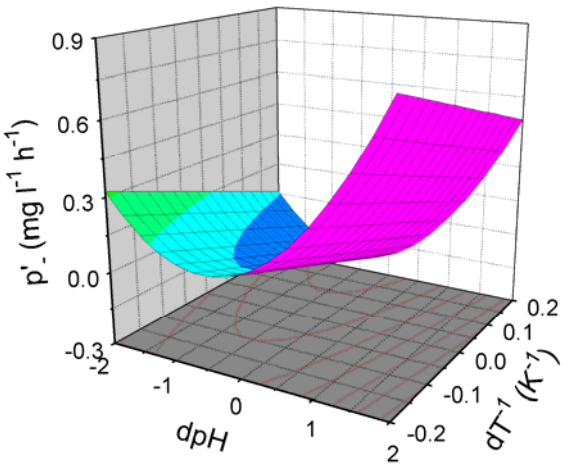

Figure 15. Response surface of ε-rhodomycinone production at 400 rpm stirring rate in the optimization of *S. peucetius* ε-rhodomycinone production. The variables are pH change and temperature change.

A pH increase with a temperature decrease seemed most beneficial for p'- (Figure 15). This implies that dynamic control strategies in the batch production of ε-rhodomycinone can increase overall process productivity. A suitable control strategy could be an initial 40 h batch phase at pH 6.5, temperature 33°C and DO control at 30%, and a subsequent 20 h dynamic phase with pH increase to 7.5, temperature decrease to 30°C and an aeration decrease to zero. The production can be completed with a batch phase in these constant environmental conditions. A control strategy using a gradual temperature decrease has also been proposed for *S. hygroscopicus* producing rapamycin (Chen *et al.*, 1999). The effects of environmental changes on the bacterial metabolism were further studied using dynamic phase continuous cultivations (4.3.2 and 4.3.3).

4.3 *S. peucetius* var. *caesius* chemostat experiments (III)

4.3.1 Steady state

Production rates of biomass (Q_X) and ε-rhodomycinone ($Q_{\varepsilon r}$) and consumption rate of glucose (Q_G) are shown in Figure 16. Yield coefficients and maintenance coefficients were evaluated from the graphs of substrate consumption and product formation vs. dilution rate (D). The coefficients for substrate consumption were Y_{XS} 0.536 g g^{-1} and m_S 0.54 mg g^{-1} h^{-1}, and for product formation Y_{PX} 12.99 mg g^{-1} and m_P 1.20 mg g^{-1} h^{-1}. A μ_{max} value of 0.10 h^{-1} could be observed (Fig. 16).

A calculated μ_{max} value with a different medium for these environmental conditions was 0.058 h^{-1}, obtained from a model from the optimization study (4.2.2). The yield coefficient Y_{XS} is reported to be 0.5 g g^{-1} for aerobic bacteria (Doran, 1995). Our result 0.53 g g^{-1} corresponds to this rather well. Other studies have reported yield coefficients for *Streptomyces* species in carbon limited conditions ranging from 0.43 to 0.63 g g^{-1} (Hilliger *et al.*, 1978; Inoue *et al.*, 1982; Melzoch *et al.*, 1997).

The maximum specific production rate of ε-rhodomycinone in batch cultivations has been around 0.32 mg g^{-1} h^{-1}. In this study, a maximum specific production rate of 0.66 mg g^{-1} h^{-1} was obtained, and an even higher, 1.20 mg g^{-1} h^{-1} maintenance associated specific production rate was estimated from the results. Similar results have been reported with other streptomycetes and other products, while comparing batch and continuous cultivation data.

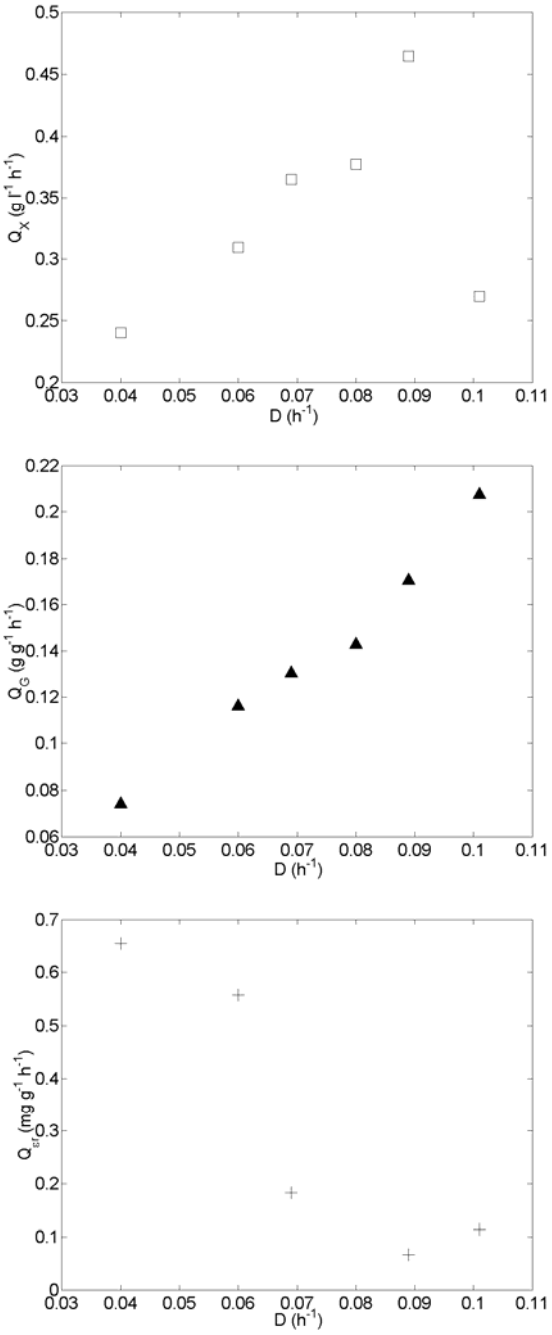

Figure 16. Steady state biomass production (□), specific glucose consumption (▲) and specific ε-rhodomycinone production (+) rates in *S. peucetius* continuous cultivations.

4.3.2 Kinetic experiments

Different environmental perturbations were conducted on a steady state chemostat of *S. peucetius*. Q_G decreased after a temperature decrease step (33 to 30^oC) and remained at the

decreased level (97% of the original flux) during the experiment. Q_X decreased at first, but regained the level prior to the change in 4 h. pH elevation (6.5 to 7.5) had no effect on Q_G. Q_X, on the other hand decreased to 84% of the original flux. Q_G remained constant also throughout the airflow reduction experiment (0.7 to 0.1 vvm). Q_X increased to 115% compared to the original value.

4.3.3 Flux analysis

Optimization results from the flux analysis are shown in Figure 17. Flows are presented as percentage of glucose feed. The flux analysis showed increasing PPP, TCA and shikimate pathway flux, and decreasing EMP and ED with decreasing μ, partly signifying transition from growth phase to production phase.

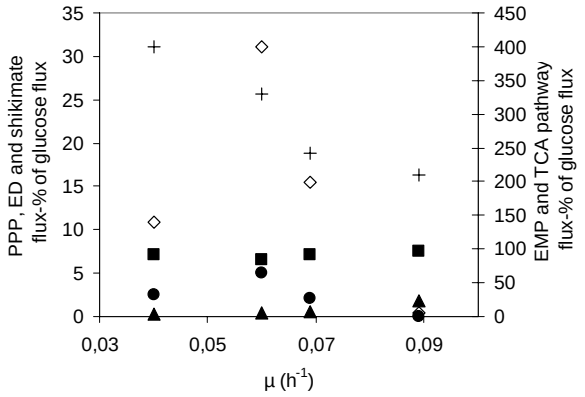

Figure 17. Metabolic fluxes of *S. peucetius* as a function of μ: PPP (◊), ED (▲), shikimate pathway (●), EMP (■) and TCA (+) as flux-% of the glucose feed flux.

Studies on other *Streptomyces* species have shown quite variable flux characteristics depending on growth vs. production. Similar behavior of PPP has been reported with *S. tenebrarius* (Borodina *et al.*, 2005a) and *S. coelicolor* (Kim *et al.*, 2004). Similar TCA behavior has been reported with *S. noursei* (Jonsbu *et al.*, 2001). The *S. coelicolor* model described the production of a growth-dependent antibiotic, which was formed through the shikimate pathway. Thus the shikimate pathway also increased in the study as the organism favored production over growth (Kim *et al.*, 2004). The decrease of ED activity was also reported with *S. tenebrarius* (Borodina *et al.*, 2005a). When examining the metabolic fluxes

with respect to the proportion of ε-rhodomycinone produced from glucose, it was found that all estimated fluxes exhibited a second order function with R^2 values over 0.98 (results not shown). This is an interesting phenomenon, which could ultimately lead to a metabolic flux driven production model.

The kinetic experiments implied that metabolic pathways undergo changes when environmental changes are applied. According to the TCA, the best effect on ε-rhodomycinone production was the pH change. In batch cultivations without pH control this elevation of pH occurs naturally. The pH increase gave a clearer result than the temperature decrease thus partly reinforcing the hypothesis presented in the optimization study (4.2.2). Studies on the combined effect, as well as other types of kinetic tests are still necessary for the validation of this hypothesis.

The use of MFA in the type of kinetic experiments conducted in this study is probably not the best way to observe the effects. MFA assumes steady state and thus a pseudo steady state assumption is made in every analysis point of the kinetic experiments. It is, however, at the moment the only possible way with reaction networks as big as the ones used in metabolic network studies. Better estimations of the fluxes can surely be obtained with kinetic models of all the reactions involved, but the amount of information required to do this is too vast for this decade.

4.4 *S. peucetius* var. *caesius* process modeling (IV)

4.4.1 Unstructured kinetic models

The cultivation growth phase was considered separately with respect to glucose and biomass values. Y_{XS} and m_S were defined in the parameter optimization as 0.536 g g^{-1} and 0.54 mg g^{-1} h^{-1}, respectively, based on data obtained from continuous cultivation experiments (4.3.1). The best biomass simulation results were obtained with the logistic and Contois models. Both the Monod and Teissier models tended to yield initial underestimates, which later on turned to overestimates of the biomass values. The best

glucose fits were obtained with the logistic and Teissier models. The Contois model overestimates residual glucose, whereas the Monod model underestimates it.

The shape of the Monod model for the modeling of *S. peucetius* var. *caesius* N47 growth was found unsuitable, a result contradicting with the model by King (1997). The fundamental assumptions of the model seem wrong according to our results, even though the proposed modeling concept was good. The same trend in model simulations compared to real data was also seen in the results obtained by King (1997), although no considerations were made on the shape issue. The logistic model, found most suitable in this study, was also used in a study modeling *S. coelicolor* growth with good results (Elibol and Mavituna, 1999).

The logistic model was chosen for use in the further investigation of the process. Lag phase and product formation were included according to equations 6 and 7. The results are shown in Figure 18. The model gave a reasonably good estimate of the measured variables.

Simulations of the kinetic process model worked out rather well. The model explanations were good, but there seems to be unexplained variation in the output, which cannot be accounted for by glucose and CO_2 data. Therefore, the kinetic model, however useful in simulations, cannot be used in the prediction of biomass and ε-rhodomycinone concentrations during arbitrary batch cultivations.

4.4.2 Neural networks

Different network configurations were tested in the estimations of biomass and ε-rhodomycinone concentrations. Data for the biomass estimation with environmental and process variables was taken from 15 cultivations in the optimization study (4.2.2). Figure 19 shows the neural network estimation of X with a 6-8-2 network as a function of growth time, S, DO, pH, T and respiration quotient (RQ).

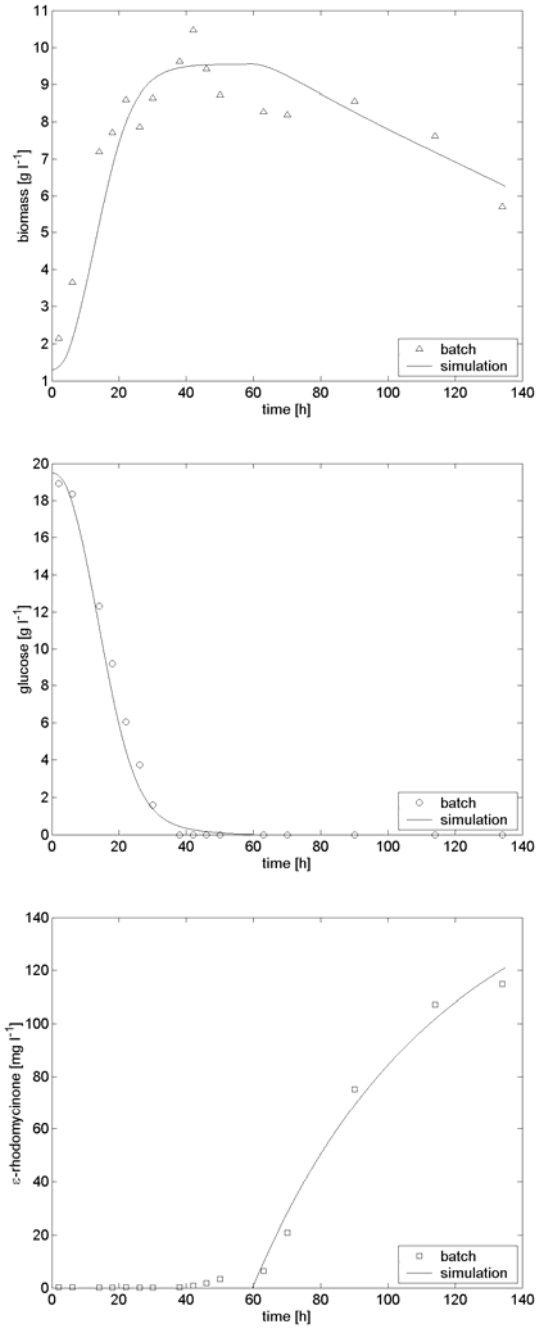

Figure 18. Biomass, glucose and ε-rhodomycinone in *S. peucetius* var. *caesius* N47 cultivation and simulation with the logistic model. The R^2 of the best fit was 0.953.

A recursive network was built to estimate the ε-rhodomycinone concentrations as functions of growth time, S, pH, T^{-1}, RQ and ln(X) (Figure 20). In these experiments, ε-rhodomycinone concentrations were measured only from the last sample at the end of the cultivation. This made it extra difficult for the product estimation. In principle, if these two

networks are used together, one can estimate both the biomass and ε-rhodomycinone concentrations from other more easily measurable variables during the cultivations.

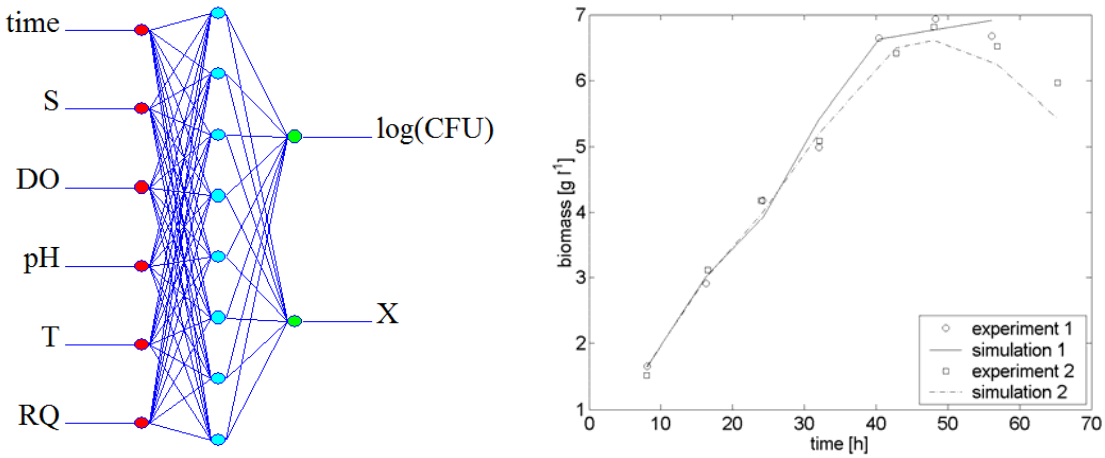

Figure 19. On the left: Neural network for the estimation of biomass (X) and biomass viability (log(CFU)) with a 6-8-2 network from *S. peucetius* var. *caesius* N47 cultivations as functions of growth time, glucose concentration (S), dissolved oxygen (DO), pH, temperature (T) and respiration quotient (RQ). Input nodes are presented in red, hidden layer nodes in cyan and output nodes in green. On the right: Model validation of X with two cultivations not shown in the training procedure. The R^2 was 0.983.

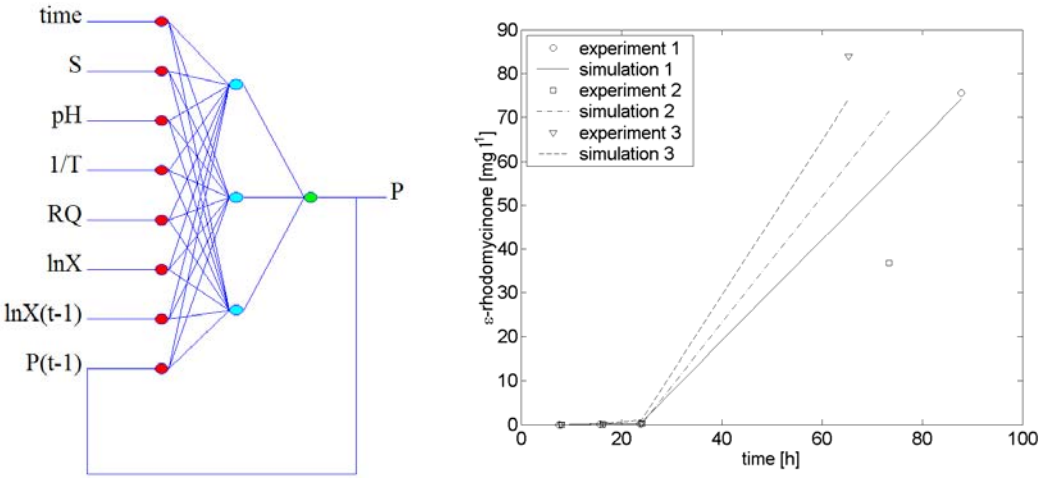

Figure 20. On the left: Neural network for the estimation of ε-rhodomycinone with a recursive 8-3-1 network from *S. peucetius* var. *caesius* N47 cultivations as functions of growth time, glucose concentration (S), pH, temperature (T^{-1}), respiratory quotient (RQ) and biomass (X). After network training the R^2 was 0.988. Input nodes are presented in red,

hidden layer nodes in cyan and output nodes in green. On the right: Model validation with three cultivations not used in the training procedure gave the R^2 value of 0.903.

The effective use of neural networks in modeling and simulation requires lots of data. The lack of product concentration values between start and end points in the optimization study cultivations used as training data caused inaccuracy in the estimation results. To enhance the estimation performance, the profiles of product formation from a couple of experiments would have been beneficial. Direct measurement of biomass from a particle containing complex medium is very difficult, but could be estimated with a neural network in a rather straightforward manner. The model can be used for capturing the dynamics of the mechanistic model for the cultivation process e.g. in model predictive bioprocess control (Mjalli and Al-Asheh, 2005). In this study, the neural networks gave more accurate data simulation patterns than the kinetic models, and were able to predict biomass and product concentrations from arbitrary batch cultivations, which is something that could not be done using kinetic models.

5 Conclusions

The modeling and optimization of bacterial processes has never been straight forward, as the nature of live biological materials makes the tasks non-trivial. In this study, good experiences were achieved with the use of experiment designs on both bacterial species. This raises possibilities for designing a straight forward procedure for the future in the optimization of bacterial processes. The possibilities are in shortened process development times and initially more optimal production processes.

The continuous cultivation technique combined with kinetic modeling reduces the need for model parameter search in the attempt to simulate batch cultivations. All the models tested and developed here could be tried out with bifidobacteria, which has also been successfully cultivated in the continuous mode. The streptomycete control strategy implied by the

optimization study could not be verified in the environmental perturbation experiments on the continuous cultivation, and further experiments are recommended for theory validation.

The results of the MFA could ultimately lead to a new concept of a process control model, but there is still work to be done in view of the measurements, e.g. HPLC and related methods. The amount of degrees of freedom in the MFA model requires so many measurements that the current laboratory capacity could not fulfill the requirement for the amount of accurately measured compounds necessary for true flux calculation and analysis. The results presented in this study were only the results of flux optimization according to a relatively small number of results accurately obtainable. These, however, showed a good possibility for application in a process control model based on MFA. Naturally, further research would be necessary for the development of this model.

The unstructured kinetic model worked well in simulating the cultivations of a complexly growing organism, but essentially lacked sufficient predictive power. The neural network, on the other hand, could predict both biomass and product formation, although two networks were required for the task, with over 90% data fitting. A further scope of study on process modeling could be the combination of kinetic models with neural networks, namely in parameter estimation, as this step is the most critical one concerning a predictive simulation model. A good, predictive simulation model could help in designing a functional dynamic control strategy with minimal laboratory testing times and thus at reduced cost. The model could also be used in predicting process outcome during production for monitoring purposes and early detection of bad batches.

6 References

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