An extended Kalman filter based control system for maximizing the biomass production in *E. coli* K1 cultivations

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Erklärung

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Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

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Jinu Mulamoottil John Hannover, März 2011

Dedication to my brother

Joji M. John

[Taken to eternal life on 28th January 2004]

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Abstract

In this work, a series of different types of cultivations are presented with an objective to maximize the biomass production and optimising the yield of the product, polysialic acid, an interesting scaffold material with extensive applications in modern tissue engineering. The production process is based on the bioreactor cultivation of *E. coli* K1 2032. In the first part of the work, the biomass and polysialic acid production during batch cultivations, glucose limited fed-batch cultivations at different fixed growth rates done with exponential feeding strategy as well as fed-batch cultivations with a model based substrate control system are illustrated. The model based control applied here is based on ordinary flow injection analysis measurement data supplemented by an extended Kalman filter which uses a Monod model for the estimation of the state variables; biomass, glucose, maximal growth rate as well as volume. Since this method is provided with a feed forward/feedback controller, the system can successfully handle the instantaneous dynamics developing during the cultivation process. The results illustrated prove that extended Kalman filter based control system is effective in maintaining the glucose concentration at a desired level for considerable duration of the process. A glucose level down to 0.05 g/L has been achieved without production of acetate as by-product. The efficiency of the controller has been proved by the calculation of uptake rate of the substrate and evolution rate of the products. A carbon balance is also made to reinforce the results. It is evident in this work that an extended Kalman filter based controlled fed-batch cultivation is the best strategy for maximizing the biomass production. Polysialic acid production is proved to be proportional to biomass production. The dependence of biomass production on the growth rate also has been studied. It is observed that as the growth rate increases, the biomass yield increases and reaches a maximum and then decreases. Thus it is established that the maximum biomass yield of 0.52 g/g and a polysialic acid yield of 0.061 g/g has been achieved at a growth rate of 0.37 h^{-1} during cultivation of *E. coli* K1 2032 at a constant glucose concentration of 0.05 g/L. Since the main emphasis of the research is the establishment of a reliable control system for maximizing the biomass production, in the second part of the thesis, extended Kalman filter controlled cultivations at different set points are analysed and discussed in detail with a focus on the problems encountered during the control process.

Key words: extended Kalman filter, fed-batch cultivations, flow injection analysis, polysialic acid, feed forward/feedback control, carbon balance, Monod model, exponential feeding

Zusammenfassung

Diese Arbeit beschreibt eine Serie verschiedener Kultivierungen zur Maximierung der Biomasse und Optimierung der Produktausbeute von Polysialinsäure. Polysialinsäure ist ein interessantes Gerüstmaterial für eine Vielzahl an Anwendungsmöglichkeiten im modernen Tissue Engineering. Der Produktionsprozess findet in einem Bioreaktor durch Kultivierung von *Escherichia coli* K1 statt. Der erste Teil der Arbeit stellt die Biomasse und Polysialinsäureproduktion während Batch-Kultivierungen dar, einschließlich glukoselimitierter Fed-Batch-Kultivierungen bei unterschiedlichen Wachstumsraten, die durch eine exponentielle Feeding-Strategie realisiert wurden und Fed-Batch-Kultivierungen, denen eine modellbasierte Substratkontrolle zu Grunde liegt. Die hier genutzte, modellbasierte Kontrolle basiert auf einer Fließ-Injektions-Analyse mit angehängtem erweitertem Kalman-Filter, dem ein Monod-Modell zur Abschätzung der Prozessvariablen Glucose, Biomasse, maximale Wachstumsrate und Volumen, zu Grunde liegt. Durch den feed forward/feedback Contoller kann das System verzögerungsfrei die Dynamik des Kultivierungssystems beherrschen. Die Ergebnisse zeigen anschaulich, dass das erweiterte Kalman-Filter-Kontroll-System die Glukosekonzentration sehr effektiv auf dem Sollwert über den Großteil der Kultivierung halten kann. Ein Glucoselevel unter 0.05 g/L garantiert keine Acetatproduktion als Nebenprodukt. Die Effektivität des Kontrollsystems wurde über die Berechnung der Substrataufnahme- und Produktherstellungsrate bestätigt, die Kohlenstoffbilanz verifiziert die Ergebnisse. Es konnte in dieser Arbeit gezeigt werden, dass der erweiterte Kalman-Filter zur Kontrolle von Fed-Batch-Kultivierungen die beste Strategie zur Maximierung der Biomasse darstellt. Es wurde gezeigt, dass Polysialinsäure proportional zur Biomasse entsteht. Die Abhängigkeit der Biomasseproduktion von der Wachstumsrate wurde daher ebenfalls untersucht. Es wurde beobachtet, dass mit ansteigender Wachstumsrate die Ausbeute an Biomasse auf ein Maximum steigt und anschließend wieder sinkt. Die maximale Ausbeute an Biomasse wurde bei 0.52 g/g mit einer Polysialinausbeute von 0.061 g/g bei einer Wachstumsrate von 0.37 h^{-1} während der Kultivierung von *E. coli* K1 2032 bei einer konstanten Glukosekonzentration von 0.05 g/L erreicht. Da der Hauptaugenmerk dieser Arbeit auf der Etablierung eines zuverlässigen Kontrollsystems zur Maximierung der Biomasseproduktion lag, wurde im zweiten Teil der Arbeit das Kontrollsystem zur Kultivierung an unterschiedlichen Sollwerten evaluiert. Diese Experimente werden insbesondere mit Bezug auf die unterschiedlichen Probleme während der Prozesskontrolle dargestellt und diskutiert.

Schlag wörte: erweitertem Kalman-Filter, Fed-Batch-Kultivierungen, Fließ-Injektions-Analyse, Polysialinsäure, feed forward/feedback Contoller, Kohlenstoffbilanz, Monod-Modell, exponentielle Feeding

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1. Introduction

Biotechnology is an extremely productive and highly innovative branch of science, evolving at a rapid pace. It is an integrated multidisciplinary domain with extensive application in sectors like food, health, agriculture and environment, which are closely related to the daily life. The living cells and the materials produced by them are the basic tools of biotechnology and it utilizes them for making advantageous products and developing new methods of production which will ultimately result in the betterment of society. Due to its diversity in the area of application, biotechnology is expected to be an effective tool for the revolutionary solution of many critical global problems encountered by the modern world like poverty, energy crisis, environmental hazards, incurable diseases and epidemics.

Polysialic acid (polySia) is a structurally unique and scientifically important biopolymer which is widely used as a scaffold material in tissue engineering (Bruns 2007, Stark 2008). It is a homo polymer of α-2, 8-linked sialic acid residue distributed in a range of bacterial capsules to brain cells. It occurs as post transitional modification of the neural cell adhesion molecule in higher vertebrates. PolySia is characterised by biocompatibility, biodegradability and remarkable chemical features for surface modifications, which makes it an outstanding chemical compound in biomedical applications (Gregoriadis 2005, Berski 2008, Steinhaus 2010). Since a lot of researches are going on all around the world about this species of compound, a reliable and standardised method for the production of polySia is of scientific interest.

PolySia is mainly isolated from encapsulated bacteria and the procedure for the isolation process is available in literature (Mcguire 1964, Uchida 1973, Oskov 1984, Rodriguez-Aparicio1988, Gonzalez-Clemente 1990, Zahn 2002). But the reported protocols focus mainly on laboratory scale preparation and isolation of colominic acid. Rode et al. tried to apply the fed-batch technique for increasing the production of polysialic acid (Rode 2008). But still it lacks a reliable and well configured method. In this context, here, a study has been carried out to establish a control system for the optimisation of polysialic acid production through maximizing the biomass production in bioreactor cultivation of *E. coli* K1.

Bioprocesses are highly dynamic in nature. Therefore, a well adapted optimal controller for bioprocess requires highly complicated regulatory network to cope up with the instantaneous

changes taking place during the process. Although commercially available process controllers are equipped with facilities to control the parameters such as pH, temperature, dissolved oxygen, stirrer speed and antifoam, automated controllers for important process parameters are still lacking. Effort is going on in the direction to establish a controller for the overall automated regulation of the bioprocess (Hitzmann 2000, Akesson 2001, Hisbullah 2002, Johnson 2002, Arndt 2005). Metabolism acts as a regulatory mechanism through which the microorganism got adapted to the changing environment which in turn depends on the substrate concentration. For example, depending on the substrate concentration, the organism undergoes changes in the uptake rate. As a consequence, the yield of the desirable product is not optimal. Changes in process parameters can also be disadvantageous when it is accompanied by the production of undesirable by-products. Acetate production by *E. coli* is a well known example. *E. coli* will produce acetate either when the available substrate concentration is high or when the oxygen supply is not sufficient. Under these circumstances, the bacteria will change from oxidative to oxido-reductive metabolism. Acetate can hinder the growth of the microorganism and results in a decrease in the amount of products especially, recombinant proteins (Bauer 1990, Bech 1990, Luli 1990, Nielsen 1992, Akesson 1999).

Considering both these facts, lack of a reliable substrate control system for the regulation of bioprocesses and the increasing demand for more amount of polySia, this work focuses on the establishment of a suitable substrate controller for the fed-batch cultivation of *E. coli* K1 2032 to maximize the biomass production and thereby optimise the production of polysialic acid. The objective might be realised in different modes of cultivations such as batch cultivations, glucose limited fed-batch cultivations using exponential feeding mode and fed-batch cultivations controlled by extended Kalman filter. More importance should be given to extended Kalman filter controlled cultivations because it is provided with a feed forward/feedback control facility which is working mainly depending on the actual conditions inside the bioreactor. An ordinary flow injection analysis (FIA) system would be used for on-line monitoring of glucose concentration during the cultivation. The performance efficiency of the controller should be evaluated by studying the uptake and production rate of substrate and products. Carbon balance can also be used to monitor the carbon flux flow during the control process. The biomass and polySia yield can be compared with respect to different growth rates to define the best condition for the maximization of biomass production and optimisation of polySia production.

2. Theory

2.1. Quantitative detection of glucose

Quantitative detection of glucose is becoming an integral part of daily life. In food industry it is essential for quality assurance procedure. The dosage of medication for metabolic disorders are decided and controlled by monitoring the glucose quantity in blood. Many biotechnological processes are also controlled on the basis of glucose concentration.

Glucose is the primary energy source for living organisms. Since glucose is easily available, inexpensive and conveniently consumed by most of the microorganisms, it is commonly used as a substrate in bacterial cultivations. Many scientific analysis methods for quantitative detection of glucose have been developed and described in literature. High performance liquid chromatography (Farine 1997), reduction/oxidation methods (Lau 2000, La Course 1993, Matsubara 2000), spectroscopic method (Liu 2009), enzymatic methods (Wu 2007, Cui 2007, Katsumata 2000, Nandakumar 1999, Weigel 1995) are some important methods among them.

In this work, enzymatic detection of glucose is used for controlling the cultivation of *E. coli*. Glucose oxidase (GOD) acts specifically with β-D-glucose and converts it into gluconic acid (Figure 1). The conversion is accompanied by consumption of oxygen and production of hydrogen peroxide.

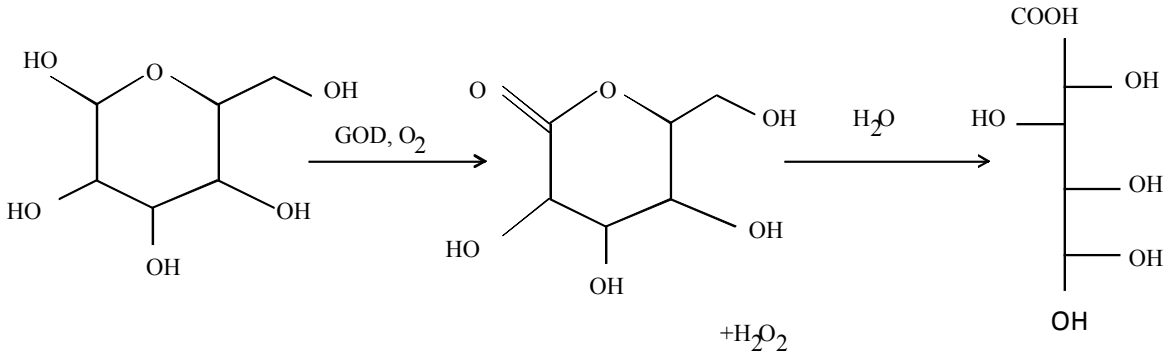

Figure 1: Conversion of glucose with GOD and oxygen to gluconolactone and hydrogen peroxide. The final product is gluconic acid.

The dissolved oxygen is measured amperometrically by a Clark electrode. The Clark electrode consists of an anode made up of silver chloride and a thin platinum wire cathode.

Only the very tip of the platinum cathode is exposed and the rest of the wire is sealed in a cylindrical epoxy. A thin membrane is placed over the tip of the platinum cathode and beneath the membrane is a narrow layer of electrolyte (4M KCl) which ensures the electrical contact between the cathode and the anode. The membrane is permeable to oxygen only. The oxygen diffusing through the membrane is reduced at the electrode and the current produced is proportional to the concentration of the dissolved oxygen. The chemical reaction taking place can be summarised as follows:

$$\textit{Anode Reaction}: \quad 4Ag + 4Cl^- \rightarrow 4AgCl + 4e^-$$

$$\textit{Cathode Reaction}: \quad O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

$$\textit{Overall Reaction}: \quad O_2 + 2H_2O + 4Ag + 4Cl^- \rightarrow 4OH^- + 4AgCl$$

The minute current produced is then amplified and recorded. The same measuring principle is used in the in-situ measurement of dissolved oxygen inside the bioreactor during cultivation run.

2.2 Metabolic pathway of *E. coli*

E. coli is a gram negative bacteria belonging to the family Enterobacteriaceae. They are found mainly in the lower intestine of warm blooded animals. One of the main characteristics of *E. coli* is that it can metabolize sugar in two different mechanisms namely respiratory or oxidative metabolism and fermentative metabolism. Another attractive feature of this species of microorganism is that it can integrate a wide range of other carbohydrates also in the metabolism. For example, glycerol can be effectively assimilated by *E. coli*. Both glycerol and glucose get converted to pyruvate in the first step and in the next step will again be converted to CO_2 and H_2O during glycolysis (Gottschalk 1988).

The first step in glycolysis is the phosphorylation of glucose. This reaction is catalyzed by a family of enzymes called hexokinases. The glucose gets converted to glucose-6-phosphate. Even though the process consumes ATP, it promotes the passage of glucose into the cells through plasma membrane transporters. Since the cell lacks transporters for glucose-6-phosphate, it obstructs the leaking out of glucose from the cells. Later it will be converted to pyruvate glycolytically. The glycolysis process yields two moles of pyruvic acid and two ATP and two NADH per mole of glucose. In the next step of the metabolism, the pyruvate undergoes a dissociation reaction into carbon dioxide with evolution of energy and NAD will

get converted to NADH. The remaining 2-C unit will get attached to the coenzyme-A to form acetyl-CoA. This acetyl-CoA will be further metabolized either by oxidative mechanism or by fermentative metabolism.

Under aerobic conditions, the acetyl-CoA will enter into Krebs cycle. It is also known as Citric acid cycle or tricarboxylic acid (TCA) cycle. The pyruvate formed via glycolysis can be completely oxidized into carbon dioxide and water in TCA cycle. Here, one mole of ATP, four moles of NADH and one mole of $FADH_2$ are formed per mole of pyruvate. The possibility for the regeneration of NAD^+ and FAD from NADH and $FADH_2$ is a must for the complete conversion of pyruvate. This mechanism is carried out by an oxidative process involving free oxygen. Therefore, complete conversion of pyruvate to carbon dioxide and water is realized only under aerobic conditions. However a total gain of 38 moles of ATP per mole of glucose is possible in respiratory metabolism (Bailey 1986).

The metabolism under anaerobic condition is called fermentative metabolism. Acetate is the main product of fermentative metabolism (Diaz-Ricci 1991). Here, pyruvate is reduced by pyruvate formate lyase to formate and acetyl coenzyme-A, which will then be converted to acetate. A replenishment mechanism also exists in the metabolic network of bacteria. One molecule of succinate is formed by the condensation of two molecules of acetate. This particular pathway is known as glyoxylate cycle (Gottschalk 1988). The accumulation of acetate hinders the growth of the organism (Nakano 1998), but the critical concentration of acetate for growth hindrance is strain specific. In addition it has an adverse effect on product formation.

A fully aerobic condition does not guarantee the complete depletion of the acetate. Acetate can be produced on excess availability of the substrate or at higher growth rates (Akesson 1999) even when a fully aerobic condition is maintained. An imbalance will be developed between the substrate uptake rate and its conversion to biomass. As a result, acetyl coenzyme-A will be withdrawn from TCA cycle to acetate formation (Marjan 2007).

2.3. Flow Injection Analysis

2.3.1. Principle of flow injection analysis

The first depiction about flow injection analysis (FIA) was made by Ruzicka and Hansen (Ruzicka 1975). Due to its simplicity and accuracy in measurement, the interest and application level of FIA is shooting up day by day. As the name indicates, it is an analytical

method based on the injection of a liquid sample (forming a sample zone) into a moving unregimented continuous stream of another suitable liquid. The sample zone will then be transported towards a detector that continuously record some parameters as it changes continuously on passage through the flow cell.

In the simplest form, a FIA system consists of a pump, which is used to propel the carrier stream through the narrow tubing, an injection port by means of which a well defined volume of a sample solution is injected into the carrier stream in a reproducible manner and a micro reactor in which the sample zone get dispersed and react with the components of the carrier stream to produce some detectable species, which will be recorded continuously. Two different mechanisms are possible for the dispersion of sample zone into the carrier stream namely, convection and axial diffusion. The use of catalysts for improved sensitivity is also applicable in suitable cases.

FIA is a dynamic analysis technique. The mixing up of sample zone into the carrier stream and conversion of sample into a detectable product takes place simultaneously. Therefore, the accuracy of the system parameter is very important for the reproducibility of the measurement result. The system parameters such as reactor length, geometric configuration, flow rate of the carrier stream, tube radius, injection volume etc. should be maintained accurately throughout the analysis. The environmental condition such as temperature must also be considered. The main advantages of FIA are its simplicity, small sample volume (below 100 μL) and flexibility in application levels. Owing to the higher sampling frequency compared to other analytical techniques, FIA is accepted as an efficient tool for on-line analysis procedures.

The transient signal observed by the detector during the passage of a sample zone is usually a peak form. The height, the width or the area under the peak contains the analytical information. Although several methods of data detection is available, peak height measurement has been the most popular one because it is the most convenient parameter to locate.

The whole process of sample/standard injection, transport, reagent addition, reaction and detection can be accomplished very rapidly, using minimum amounts of sample and reagents, and with excellent reproducibility (e.g. coefficient of variation, CV, generally < 2 %). Although complete equilibrium may not be achieved during this process, quantization is

possible because both standards and samples are dispersed to the same extent and processed in an identical manner.

Thus the attractive features of FIA are its sensitivity, selectivity, precision, rapidity, simplicity, economy and versatility.

2.3.2. FIA with immobilized enzyme

A FIA system with immobilized enzyme cartridge represents a detection system in the stationary phase. It is based on the same principle as described in chapter 2.1 i.e., using glucose oxidase enzyme solution. Here the enzyme GOD is immobilized on a polymer cartridge. The sample stream is injected into the carrier stream by an injector and passes through the enzyme cartridge. There, the enzymatic conversion of glucose takes place and the used oxygen concentration is measured by the Clarks electrode as explained in chapter 2.1. (Page No. 3).

This type of enzyme system indicates a higher dispersion in the enzyme cartridge. A negative point regarding this type of enzyme cartridge is that the samples should be completely cell and particle free, otherwise the cartridge get damaged soon.

2.3.3. FIA with enzyme solution

The basic principle of quantitative measurement of glucose using FIA with enzyme solution is the same as explained in chapter 2.1 (Page 3). In this technique also the oxygen consumed during the reaction is measured.

A continuous stream of a suitable carrier is maintained throughout the measurement process which represents an unsegmented flow. The sample to be measured is injected directly into the carrier stream. As required in immobilized enzyme system, a cartridge is not necessary in this experimental set up. The GOD enzyme solution is also injected into the carrier stream just before the sample zone. The time interval between the two injections is decided by the carrier flow rate, distance between the two injectors as well as the inner diameter of the tube used for connection. The enzyme and the sample zone will then pass through the zig zag reaction coil in which the intermixing and enzymatic conversion into detectable species takes place. The time delay of individual measurement depends on the time taken by the sample to pass through the reaction coil and the distance between the injector and the bioreactor. A

longer reaction coil represents a better mixing up, but it will result in an increased time delay which is not desirable in process control.

There are so many advantages for this type of FIA in comparison to the system with immobilized enzymes. First of all the problem of damage to the cartridge, which is a problem encountered in the other one can be avoided. Also, the concentration of the GOD solution can be varied according to the concentration range of the substrate to be measured. But, still FIA come across some problems. The air bubble is one of the main problems. Even very small air bubbles can disturb the electrode and will give odd measurement data. Even if sampling module is used for the on-line sampling process, the possibility of air bubbles cannot be ruled out when the viscosity of the medium is high. Care should be taken to see that the injectors and connection tubing are clean enough so that the flow will not be disturbed. If the sensitivity of the electrode is going extremely lower, one could infer that the electrode membrane is damaged or there isn't enough electrolyte inside the membrane. So changing the membrane at regular intervals will make the system more stable.

2.4. Bioprocess control and controller types

A bioprocess control is a systematic procedure in which (Unbehausen 1994) the output variable *x (t)* is continuously measured and compared with a previously determined set point *w (t).* The difference between the output variable and the set point is the control deviation ($x_w(t)$), represented by equation [1].

$$x_w(t) = x(t) - w(t) \qquad [1]$$

The simplest controller is a two point controller or on/off controller. This can be easily implemented; nevertheless it is proved to be functioning satisfactorily in many control processes. In this type of controllers, the controller will start functioning when the measurement deviation exceeds certain value. When the deviation becomes negligibly small or zero, then the controller will return to the off mode. This continuous on and off mode switching behavior gives a pulsation effect to the output variable so that it found seldom application in industrial field.

The efficiency of a bioprocess control is determined by the response of the controller to the instant fluctuations in the process conditions. Features of the control algorithm programmed into the system are the sole factor which determines the qualitative as well as quantitative response of the control. As the name signifies, a control algorithm is a set of sequential

mathematical expressions which forms the basis for the attainment of a successful and effective process control. They are often programmed as equations with control output as a function of the difference between the measured signal and the intended set point. Since the resultant control output is dependent on the actual measured signal, most of the controllers can function as feedback controllers.

The type of algorithm most often used in industry is coined by the three terms of PID controllers. The PID controller is composed of three elements, P: proportional, I: integral and D: derivative/differential. The collective objective of these three functions is to provide a rapid reflex to the process deviation and to scale the response in such a way to give a smooth control action. The key characteristics of PID controller are:

- Proportional control provides an output which is proportional to the variation of the measured variable to the set point
- Integral control is used to improve the effect of proportional control, helping to bring the measured variable to set point faster by minimizing the integral of control error.
- Derivative action is used to improve the effect of proportional control too, this time by estimating the slope of the control deviation with time and minimizing it.

Combining all the above mentioned characteristics, the PID controller obeys the following equation:

$$y(t) = K\left[\; e(t) + \frac{1}{T_I}\int_0^t e(\tau)\,d\tau + T_D\frac{de}{dt}\right] \qquad [2]$$

P- part I- part D- part

Here, $y(t)$ is the correction variable calculated by the controller and $e(\tau)$ is the control error, K is the amplification factor, T_I the integration time constant and T_D the derivative time constant.

Eqn. [2] is valid for an analog controller. The controller can also be written as digital algorithm. Then equation [2] will get converted to equation [3].

$$u(k) = K\left[\; e(k) + \frac{T_A}{T_I}\sum_{i=0}^{k-1} e(i) + T_D\frac{e(k)-e(k-1)}{T_A}\right] \qquad [3]$$

P- part I- part D- part

Here, $u(k)$ is the control value, $e(k)$ is the deviation from the set point at time point t = kT$_A$ [T$_A$ is the time interval between two measurements]. For software based implementation of the PID controller, one should use a recursive algorithm.

$$\Delta u = u(k) - u(k-1) = q_0 e(k) + q_1 e(k-1) + q_2 e(k-2) \quad [4]$$

The parameters q_0, q_1 and q_2 can be regarded as directly related to the P, I and D parts of the controller. Therefore, the values can be derived from equation [4]

$$q_0 = K\left(1 + \frac{T_D}{T_A}\right) \quad [5]$$

$$q_1 = -K\left(1 + 2\frac{T_D}{T_A} - \frac{T_A}{T_I}\right) \quad [6]$$

$$q_2 = K\frac{T_D}{T_A} \quad [7]$$

The commercial waves of the modern technological world insist on upgrading the efficiency of the bioprocess control and an increase in the degree of process automation. In economic point of view optimum production is the main focus. For the achievement of best possible growth rate, the environmental conditions should be maintained at an optimal level. Besides the high process dynamics, the non-linear behavior of the bioprocess also should be handled successfully by the controller. But the lack of a meticulous model for monitoring such problem still exists. Hence such systems are extremely complex. Also the measurement of process variables may be difficult, indirect or sometimes impossible. In such cases actual state estimator like Kalman filter can be adopted (Bastin 1990). There are so many controllers available for maintaining important process factors such as pH, temperature and dissolved oxygen. These are not only the necessary process parameters to be controlled. For example, the dissolved oxygen concentration in a bioreactor can be controlled at a definite set point by the controlled feeding of the substrate, when the aeration rate and stirrer speed remains constant (Kleist 2002). Another example is the control of feeding rate by calorimetric method (Randolph 1990). Kollecker designed another method for bioprocess control by applying 2D fluorescence spectroscopy (Kollecker 2006). A substrate control by measurement of dissolved oxygen concentration is also described in literature (Akesson 1999, Mare De 2003). The idea of controlling the bioprocess by taking limited substrate as control parameter is not

new (Fuld 1957). Since high instrumentation as well as regulatory techniques is necessary for this purpose, these types of controllers are seldom found in literature (Kleman 1991).

2. 5. Bioprocess model

Kinetics forms the foundation for the model based description of a bioprocess. The cell growth and other dynamic properties of the microorganism constitute the micro kinetics of a bioprocess whereas the reactor type, transport action and mixing phenomena can be considered under macro kinetics. Thus, a bioprocess model can be considered as the detailed description of the bioprocess as a sum of its micro as well as macro kinetics. It provides a deep insight into the process in its totality.

Depending on the type of microorganism under consideration, the growth can be considered either as population growth (due to cell division) or cell growth (due to increase in size). The process model can vary in its degree of complexity also. It can be roughly macroscopic or can be detailed involving metabolism and transport phenomena occurring during the bioprocess.

The cell growth kinetics of a bioprocess can be described mathematically at different levels which vary in its degree of complexity as:

- Molecular level by considering each individual steps in the cellular metabolism
- In cellular level by considering the micro kinetics of the cellular metabolism as a whole
- In population level by considering the cells as a uniform population
- In macro kinetic level by considering significant variables of the cell metabolism.

2.5.1. Description of bioprocess through models

Each individual cell is a multi-component system which is usually non-homogeneous even at the individual cell level. Cell to cell heterogeneity is a common phenomenon in a growing cell population because many self-regulating chemical reactions occur concurrently in each cell. That is to say that on the single-cell level, the different cells in a population at a given point of time varies with respect to age as well as with respect to chemical activity. Due to this complexity, the formulation of a kinetic model including all the influencing factors becomes very challenging. Medium composition is an important environmental factor which influences the growth of the organism. The formulation of the cultivation medium is carried out in such a way that all the components, except one are present at adequately high

concentrations so that the variation in their concentration does not affect the overall rate significantly. Thus, a single component turns into the rate limiting nutrient, the effect of which should be considered while analysing the effect of medium composition on the cell kinetics.

The classification of bioprocess models can be done in two perspectives. The first one is in accordance with the number of components used in the cellular representations. It is also possible to classify bioprocess model in terms of whether or not the cells are considered as the heterogeneous collection of discrete entities or as some kind of average cell which becomes almost the same conceptually as component in the solution. Multi-component cellular representations are called structured and the single component representations are designated as unstructured. A segregated view point considers cells as discrete heterogeneous cells while an unsegregated perspective considers only average cellular properties. This type of classification was first presented by Arnold Fredrickson (Fredrickson 1970) and Henry Tsuchiya (Tsuchiya 1966) and the span of each model is illustrated in Figure 2.

The simplest model is unstructured unsegregated model which can be considered as the most idealized case. But the realistic case is a segregated structured model. It is possible to introduce the ‘average cell approximation’, if the heterogenic nature of the individual cells does not affect the kinetic process of interest to a large extent. In a balanced growth state, the average cellular composition is not affected by proliferation of the population. In this situation, the multi-component nature of the cells can be ignored and an idealized unsegregated unstructured model can be used for describing the growth of cellular population.

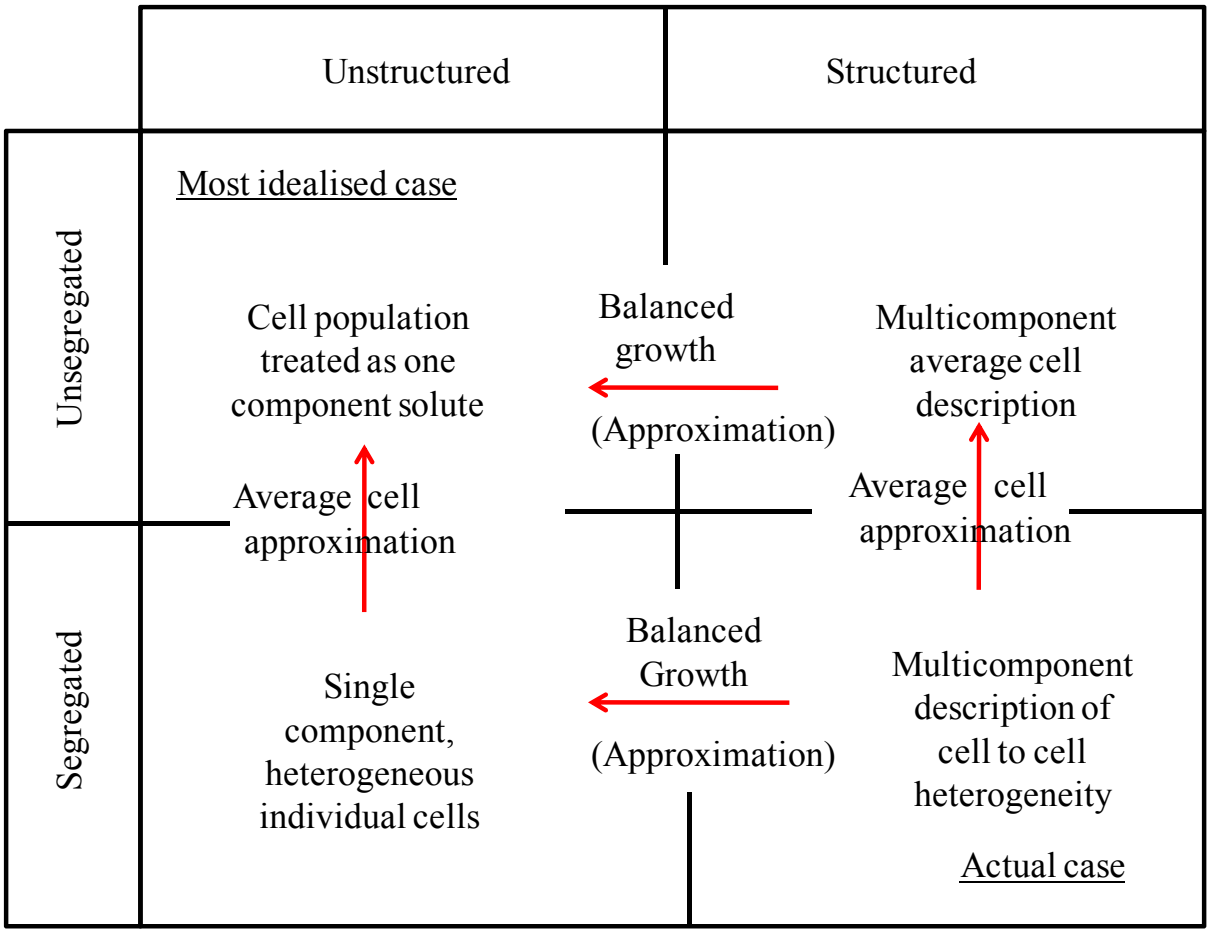

Figure 2: Different perspectives for cell population kinetic representations (from Bailey 1986).

In a bioprocess, the growth rate of the microorganism can be described in terms of the biomass concentration X and specific growth rate μ as:

$$r_X = \frac{dX(t)}{dt} = \mu X \quad [8]$$

where, r_X is the rate of biomass formation. The rate of substrate consumption is given by

$$-r_S = \frac{1}{Y_{X/S}} r_X \quad [9]$$

Here, $Y_{X/S}$ is the yield of biomass with respect to the consumed substrate.

The structured model is very complex. The adequacy of such models for designing the control system should be verified in each individual case. Therefore, an unstructured unsegregated model is desirable for the designing of a control system. This represents the

idealized model and it is capable of describing the microorganism under specified conditions with reasonable precision. This increases the reliability of the control system under different growth conditions.

2.5.2. Monod model

Monod model is an unstructured macro kinetic model which has been most frequently used in microbiology. It was put forward by Nobel laureate J. Monod (Monod 1949, Pirt 1975, Koch 1997, Koverova-Kovar 1998). Michelis Menten kinetics forms the basis of the model. The kinetic parameters used in this model for the description of bacterial growth are the maximum growth rate μ_{max} and the Monod saturation constant K_m. Monod model can be formulated mathematically as:

$$\mu = \mu_{max} \frac{S}{K_m + S} \quad [10]$$

Here, S is the substrate concentration. In the Monod model, K_m is that value of the limiting substrate concentration at which the specific growth rate is half its maximum value. Thus by combining equation [8] and [10], the biomass growth rate can be expressed mathematically as

$$r_X = \frac{dX(t)}{dt} = \mu X = \mu_{max} \frac{S}{K_m + S} X \quad [11]$$

In the same way, the substrate consumption rate can be expressed as:

$$-r_S = \frac{1}{Y_{X/S}} \mu_{max} \frac{S}{K_m + S} X \quad [12]$$

2.5.3. Monod model for fed-batch processes in ideal stirred tank reactor

Continuous stirred tank reactor (CSTR) represents the most ideal reactor type. The basic assumption of these types of reactors is that absolute mixing is possible inside the bioreactor by continuous agitation. The possibility of generation of a temperature gradient or concentration gradient can be ignored in such cases. Consequently, only the changes in terms of the volume exchange as well as the chemical reactions inside the reactor should be considered while making a mass balance in a CSTR. The mass balance for an ideal CSTR can be summarized as the following set of equations

$$\frac{dX(t)}{dt} = r_X - \frac{\dot{V}(t)_{feed}}{V(t)} X(t) + \frac{\dot{V}_{Sample}}{V(t)} X(t) \quad [13]$$

$$\frac{dS(t)}{dt} = -r_S + \left(S_0 - S(t)\right) \frac{\dot{V}(t)_{feed}}{V(t)} \quad [14]$$

$$\frac{dV(t)}{dt} = \dot{V}(t)_{feed} - \dot{V}_{Sample} \quad [15]$$

where,

$X(t)$ is the biomass concentration at time *t* [g L^{-1}]

r_X is the rate of biomass formation [g L^{-1} h^{-1}]

$\dot{V}(t)_{feed}$ is the feed rate at time *t* [L h^{-1}]

$V(t)$ is the reactor volume at time *t* [L]

$\dot{V}_{Sample}$ is the on-line sample flow rate [L h^{-1}]

$-r_S$ is the rate of consumption of substrate *S* [g L^{-1} h^{-1}]

S_0 is the concentration of feed solution [g L^{-1}]

$S(t)$ is the substrate concentration at time *t* [g L^{-1}]

In the above mentioned equation set, the production rate of biomass alone is considered and the production rate is proportional to the existing biomass. The proportionality factor is the specific growth rate μ (Refer equation [8]). Likewise, the rate of substrate consumption is inversely proportional to the yield factor $Y_{X/S}$ (Refer equation [9]). Substituting equation [11] in equation [13] and equation [12] in equation [14], the following model equations can be formulated:

$$\frac{dX(t)}{dt} = \mu_{max}\frac{S(t)}{K_m + S(t)}X(t) - \frac{\dot{V}(t)_{feed}}{V(t)}X(t) + \frac{\dot{V}_{Sample}}{V(t)}X(t) \quad [16]$$

$$\frac{dS(t)}{dt} = -\mu_{max}\frac{S(t)}{K_m + S(t)} \cdot \frac{X(t)}{Y_{X/S}} + \left(S_0 - S(t)\right)\frac{\dot{V}(t)_{feed}}{V(t)} \quad [17]$$

$$\frac{dV(t)}{dt} = \dot{V}(t)_{feed} - \dot{V}_{Sample} \quad [18]$$

This differential equation system describes the cell growth in a fed-batch stirred tank reactor.

2.6. Kalman filter

Kalman filter is a computational algorithm which is used to estimate the state of a dynamic system using continuous, time varying and noisy measurement signals. Its purpose is to minimize the mean square error between the desired output and the actual output. Usually it is applicable to linear systems (Riberio 2004).

But in actual situations, the system state dynamics and the observation state dynamics may not be always linear. Under such non-linear conditions, the probability density function that provide minimum mean square estimate becomes non-Gaussian. These non-Gaussian functions can be propagated and their mean value will be evaluated by an optimal non-linear filter. But this imposes a high computational load to the system. A worthwhile methodology to solve this problem is the implementation of extended Kalman filter (EKF), although it is a non-optimal approach. The EKF successfully applies a Kalman filter for an approximate linearization of the original non-linear filter dynamics.

In this work, the continuous extended Kalman filter uses the following equations for the continuous prediction of the state variables.

$$\frac{dx(t)}{dt} = f(x(t), u(t), t) + z(t) \quad [19]$$

$$y(t_i) = h\big(x(t_i)\big) + S(t_i) \quad [20]$$

Here, $x(t)$ is the system state variable at continuous time, $x(t_i)$ is the system variable at discrete time step, f is the function describing the change of the system state variable with the time, $u(t)$ is the input variable, $z(t)$ is the process noise, $y(t_i)$ is the measurement value at time step t_i, h is the measurement function and $S(t_i)$ is the measurement noise. The average value of the process noise and measurement noise is taken as zero since they are

considered as random noise. The intensity of noise can be satisfactorily explained by the process noise spectral density matrix *Q* and the measurement noise covariance matrix *R*.

$$Q * \delta(\tau) \;=\; E\{z(t).z^T(t+\tau)\} \qquad [21]$$

$$R \;=\; E\left\{s(t_i).s^T(t_j)\right\} \qquad [22]$$

Here, E{} is the expectation value from {}. Since each individual measurement is independent, the measurement noise covariance matrix becomes a diagonal matrix. The intensity of measurement noise covariance matrix can be specified by assigning a definite value to the variances in R, which gives an insight to the measurement quality of the model. The process error covariance matrix Q gives an index of the quality of the process model. The estimation error covariance matrix *P* illustrates the actual inaccuracy of the state vector $x(t)$.

$$P_{ii}(t) \;=\; E\left\{(x_j(t) - \hat{x}_j(t))^2\right\} \qquad [23]$$

where $x_j(t)$ is the real value and $\hat{x}_j(t)$ is the estimated value of the state variable. The estimation error covariance matrix turns up to a new value continuously in accordance with the respective measurements as equation [24].

$$\frac{dP}{dt} \;=\; F(t)P(t) + P(t)F^T(t) + Q \qquad [24]$$

Here, $F(t)$ is the Jacobean matrix of $f(x(t),u(t),t)$ which is obtained from the process model .Whenever a new measurement is available, filtering will be carried out by the Kalman filter and new values of state variables and estimation error covariance at time t_i will be calculated. Here, the Kalman-filter-gain *K* (t_i) will be calculated with the estimation error covariance of the previous prediction in which H (t_i) is the Jacobean matrix from $h\big(x(t_i)\big)$.

$$K(t_i) \;=\; P(t_i)H^T(t_i)[H(t_i)P(t_i)H^T(t_i) + R]^{-1} \qquad [25]$$

The EKF calculates Kalman-filter-gain to minimize the error covariance of estimation (Hitzmann 2000). The estimation error covariance matrix and measurement error covariance are influenced by the Kalman–filter-gain in such a way that with smaller measurement error, the estimated values will be oriented more towards the real measurement. If the circumstances got reversed, then the newly estimated value will be more directing towards the prediction. With the new Kalman-filter-gain matrix, the new estimation of the process variable will be done according to equation [26].

$$\hat{X}_{new}(t_i) \;=\; \hat{X}(t_i) + K(t_i).[y(t_i) - \; h(\hat{x}(t_i), t_i)] \qquad [26]$$

$\hat{X}_{new}(t_i)$ is the filtered value of the estimated state variable, $\hat{X}(t_i)$ is the estimated value of the state variable before filtering and $h(\hat{x}(t_i), t_i)$ is the expected measurement value calculated by the estimated values of the state variable before filtering (Hüll 2007).

The new estimation error covariance $P_{new}(t_i)$ follows equation [27], with *I* as unit matrix.

$$P_{new}(t_i) \;=\; [I - K(t_i)H(t_i)]\, P(t_i)\, [I - K(t_i)H(t_i)]^T + \; K(t_i)RK^T(t_i) \qquad [27]$$

The state equation for the cultivations under consideration can be described by the differential equation system given as equation [28]. For the integration of this differential equation system, 4th order Runge-Kutta method is employed.

$$f(x(t), u(t), t) + \; z(t) = \begin{bmatrix} \mu_{max} \frac{S(t)}{K_m + S(t)} \; X(t) - \frac{\dot{V}(t)_{feed}}{V(t)} \; X(t) + \frac{\dot{V}_{Sample}}{V(t)} \; X(t) \\ - \mu_{max} \frac{S(t)}{K_m + S(t)} \cdot \frac{X(t)}{Y_{X/S}} + \left(S_0 - \; S(t)\right) \frac{\dot{V}(t)_{feed}}{V(t)} \\ 0 \\ \dot{V}(t)_{\;feed} - \; \dot{V}_{Sample} \end{bmatrix} + \begin{bmatrix} z_x \\ z_S \\ z_\mu \\ z_v \end{bmatrix}$$

$$= \begin{bmatrix} \frac{dX}{dt} \\ \frac{dS}{dt} \\ \frac{d\mu_{max}}{dt} \\ \frac{dV}{dt} \end{bmatrix} \qquad [28]$$

The equation $\frac{d\mu_{max}}{dt} = 0$ is added as an additional state variable equation so that the Kalman filter can estimate its values according to the measurements.

The discrete continuous extended Kalman filter provides a continuous estimation of the system state variables by using the available measurement sequences. The prediction of the state variables will be carried out during the time interval in between two successive measurements. Whenever a new measurement is available, the state variables and estimation error covariance will be recalculated instantly. When the intended cultivations have to be performed under predetermined constant substrate concentrations, the time delay of concentration measurement is a major problem. But the extended Kalman filter used in here is also provided with an option to compensate the time delay during the measurements (Klockow 2008).

2.7. Applications of extended Kalman filter in bioprocesses

Extended Kalman filter found wide spread application in the prediction of state variables in a dynamic bioprocess system. References about the application of extended Kalman filter in bioprocess control are found extensively in literature. A brief overview of the literature survey is given in the following paragraphs. Elaborated explanation of the mathematical algorithm has already been published (Kalman 1960, Maybeck 1979, Balakrishnan 1987, Brammer 1989, Vandeginste 1998, Plessis 2000, Gelb 2001, Welch 2001).

A pioneer contribution to the application of EKF in bioprocess found in the literature was made by Svreck et al. (Svreck 1974). They used EKF in simulations to control the biomass over the desired variation in dilution rate.

An effort to control the dissolved oxygen inside the culture broth at a constant level by varying the feed rate is explained in the literature by Dubach and Märkl (Dubach and Märkl 1992). In their contribution, during the fed-batch cultivation of *E. coli* on glycerol, pO_2 was used as the measurement and control variable. Pol et al. (Pol 1995) described the use of EKF for the regulation of substrate concentration during the fed-batch cultivation of hybridoma cells. The concentration of the substrates, glucose and glutamine measured on-line using a multi-channel FIA is used as the control variable. Kelle et al. (Kelle 1996) explained the utilization of EKF for controlling glucose concentration during the fed-batch cultivation of *Corynebacterium glutamicum*. The successful adoption of the controller provided an efficient method for the cost effective production of L-isoleucine. Weuster-Botz et al. (Weuster- Botz 1997) realised a method to control the glucose concentration during the fed-batch cultivation of *Corynebacterium glutamicum*. They had successfully used the EKF control for maintaining the glucose concentration at a set point of 9 g/L. The application of EKF control

for the regulation of feed rate during the fed-batch cultivation of *Ralstonia eutropha* was discussed by Leonard et al. (Leonard 1999). They had used the spectroscopic method for the measurement of glucose concentration. Fed-batch cultivation of S. *cerevisiae* at a set point of 0.5 g/L has been explained by Zigova et al. (Zigova 1999) where RQ is used as the measurement variable. Hitzmann et al. discussed the application of EKF for the controlled fed-batch cultivation of S. *cerevisiae* at a glucose set point concentration of 0.5 g/L. For the measurement of glucose concentration which is used as the control parameter, they used a special FIA system (Hitzmann 2000). Control of glucose concentration at a set point of 0.07 g/L in S. *cerevisiae* fed-batch cultivation has been described by Lucena et al. (Lucena 2001). The contribution of Michael Arndt (Arndt 2001, Arndt 2004) in the implementation of Kalman filter for bioprocess cannot be ignored. He successfully used EKF for the control of glucose concentration at set points of 0.05 g/L and 0.08 g/L during the fed-batch cultivation of S. *cerevisiae*. Arndt (Arndt 2005) again used the EKF for the optimisation of the production process of phytase by *E. coli* cultivations. He used a fast glucose measurement FIA for maintaining the glucose concentration at 0.2 g/L during the fed-batch phase. Using growth rate as control variable and biomass as measurement variable, Jenzsch et al. (Jenzsch 2005) used EKF in fed-batch cultivation of *E. coli*. Klockow (Klockow 2008) used EKF for controlling the substrate concentration at set points 0.5 g/L and 0.07 g/L during the fed-batch cultivation of S. *cerevisiae*.

A large number of contributions illustrating the application of EKF for prediction both in simulations and cultivations can be found in the published scientific works and a few among them are pointed out below to get an idea about the possibilities of EKF applications in this sphere. Stephanopoulos et al. (Stephanopoulos 1984) made contributions both in theoretical as well as application level of EKF. Gudi et al. (Ghudi 1994) illustrated the use of EKF for the estimation of observable process parameters such as net growth rate by using the measurable properties, biomass as well as carbon dioxide production rate for the fed-batch fermentation of *Streptomyces clavuigerus*. For the estimation of consumed amount of NaOH and CO_2 in the exhaust gas during the fed-batch cultivation of *E. coli* Hilaly et al. (Hilaly 1994) applied an extended Kalman filter. Grosse-Uhlmann and Bley applied EKF for the prediction of biomass in the fed-batch cultivation of *Methylyobacterium rhodesianum* on methanol (Grosse-Uhlmann 1999). Biomass measured as optical density was used as the measurement variable in their contribution.

Soons et al. used extended Kalman filter for the on-line estimation of biomass, specific growth rate and oxygen transfer coefficient (Soons 2008). The frequent oxygen uptake rate (on-line) and the non-frequent biomass (off-line) were used for the online estimation procedure. Three types of DSP algorithms have been used in the bioreactor cultivation of yeast by Mangesh et al. (Mangesh 2008). They have used extended Luenberger observer (ELO) based, extended Kalman filter (EKF) based and adaptive state estimator (ASE) based DSP for estimating the state variables in yeast fed-batch cultivations and the results proved the advantage of EKF based approach with minimum estimation error over the other two approaches, even though the simplest approach is ASE based one. Lillacci et al. (Lillacci 2010) applied extended Kalman filter for the parameter selection in two biological models; model of heat shock response in *E. coli* and a model of a synthetic gene regulation system. The extended Kalman filter has been used in combination with a posteriori identifiability test to confirm the reliability of the estimated state variable. An orthogonal collocation dynamic optimisation method was adopted by Yazdani et al. for the optimisation of batch processes to obtain maximum product yield (Yazdani 2010) by controlling the inlet jacket temperature of the batch reactor. The uncertain process parameters were estimated by extended Kalman filter and the simulation results proved the improvement of batch reactor performance. Belmonte-Izquierdo proposed a recurrent high order neural observer for anaerobic processes. An extended Kalman filter based algorithm is implemented which is used to estimate biomass, substrate and inorganic carbon in the cultivation system. The simulation results are confirmed in a laboratory scale experiment. (Belmonte-Izquierdo 2010).

2.8. Uptake and production rates

The calculation of uptake and production rates of the extra cellular materials provides much clear insight into the metabolism of the microorganism. It helps in explaining the metabolic changes occurring during the growth of the microorganism. Equations [29] to [32] show the calculation of these rates. Especially, during the balancing the production rate of volatile components like acetate must be considered because there could be a chance of escape of these compounds through the exhaust gas (Mauch 2000).

Specific glucose uptake rate

$$sS_{UR} = \frac{\dot{V}_{feed} \cdot S_{feed}}{DCW \cdot V_R \cdot M_S} \quad \text{if} \quad \frac{dS}{dt} = 0 \qquad [29]$$

sS_{UR} Glucose uptake rate [mol/h]

V_R Volume of the culture broth inside the bioreactor [L]

S_{feed} Glucose concentration in the feed solution [g/L]

DCW Dry cell weight [g/L]

M_S Molar mass of glucose [g/mol]

$\dot{V}_{feed}$ Feed rate [L/h]

Specific oxygen transfer rate

$$sOTR = \frac{90\ {}^{L}/_{h}}{V_R . 22.41\ {}^{L}/_{mol} . DCW} \left[\frac{O_{2,Start}}{100} - O_2 . \frac{100 - O_{2,Start} - CO_{2,Start}}{100 - O_2 - CO_2} \right] \qquad [30]$$

$sOTR$ Specific oxygen uptake rate [mol/g_{DCW}/h]

$O_{2,Start}$ Oxygen concentration at reactor inlet [%]

O_2 Oxygen concentration in the exhaust gas [%]

$CO_{2,Start}$ Carbondioxide concentration at the reactor inlet [%]

CO_2 Carbondioxide concentration in the exhaust gas [%]

Specific carbon dioxide production rate

$$sCPR = \frac{90\ {}^{L}/_{h}}{V_R . 22.41\ {}^{L}/_{mol} . DCW} \left[\frac{CO_2}{100} . \frac{100 - O_{2,Start} - CO_{2,Start}}{100 - O_2 - CO_2} - \frac{CO_{2,Start}}{100}\right] \quad [31]$$

$sCPR$ Specific carbon dioxide production rate [mol/g_{DCW}/h]

Biomass production rate

$$\mathrm{DCW_{PR}} = \frac{\Delta[\mathrm{DCW} . V_R]}{\Delta \mathrm{t}} \quad [32]$$

$\mathrm{DCW_{PR}}$ Biomass Production Rate [g/h]

Δ Change in dry cell weight [g]

Δt Time [h]

2.9. Carbon balance

Carbon balance is an effective tool for screening the course of carbon flux flow during a bioprocess. The microorganism consumes the added carbon source and converts it into the end products of the process through metabolism. A part of the applied substrate will get converted to biomass. The rest will be distributed to the other by-products including carbon dioxide depending on the applied process conditions. A quantitative resolution of the applied carbon source in the beginning of the cultivation and the carbon output at the end of the cultivation are made. The ratio of these two values is called carbon balance or carbon recovery index. The reliability of the measurements can be making out from the numerical value of this index. Higher the index, higher will be the measurement efficiency. While making a carbon balance, the following points must be considered to make it more precise.

- In the beginning of the carbon balancing, all the provided carbon resources (carbon source from medium, biomass and acetate) should be considered.

- The up taken amount of carbon from the medium as well as carbon from the feed solution.
- At the end of the balance, the amount of carbon existing in the medium (carbon source in the medium, biomass and acetate).
- The amount of carbon in the form of biomass, glucose and acetate taken while sampling.
- Carbon dioxide in the exhaust gas also must be considered at the end of the carbon balance.
- The amount of acetate escaping through the exhaust gas.

The carbon content of the biomass should be analysed for executing proper carbon balance.

3. Materials and methods

3.1. Structure and characterisation of the FIA system

The on-line detection of glucose concentration during the fed-batch cultivations is carried out by a flow injection analysis system. The basic principle of glucose detection is the same as explained in chapter 2.1 (Page 3). For the enzymatic conversion of glucose, glucose oxidase enzyme solution is used in this work. The hardware is based on a commercial FIA system (ANASYSCON Instrumentelle Analysentechnik GmbH, Hannover), which is modified to be adapted to the current conditions. The FIA system mainly consists of the following parts:

- Three peristaltic pumps (ACCU CP10, SciLog, USA with pump head Masterflex Coleparmer Instrument Co., Illinois) for passing on the buffer, glucose oxidase enzyme solution and sample to be measured into the injection system.
- One Selector valve (K-6 valve drive with 7 port/ 1 channel valve drive, KNAUER, Berlin) for switching in between different standard solutions during calibration.
- Two injection valves (K-6 valve drive with 6 ports/ 3 channel injection valve, KNAUER, Berlin), for the injection of sample and glucose oxidase enzyme solution into the buffer flow.
- An amplifier and a Clark electrode (ANASYSCON Instrumentelle Analysentechnik GmbH, Hannover) integrated in acryl housing.

A continuous carrier stream should be maintained in the FIA system throughout the measurement. Here, buffer solution is used as the carrier and it is formulated according to the composition given in Appendix A.2. The commercial glucose oxidase (GOD) from *Aspergilus niger* (Fluka, Germany) is used for the preparation of GOD solution. FIA buffer is used as the solvent and calculated amount of GOD is dissolved into it to prepare stock GOD solution. The concentration of the GOD solution must be optimized to get a reliable and reproducible signal of the measurement data. Concentration ranging from 100000 U/L to 800000 U/L were prepared and tested for measurements in the desired glucose concentration range. GOD concentration of 500000 U/L was selected as the standard concentration since it gave the minimum standard deviation for all concentrations of glucose under consideration.

The sample to be measured has been withdrawn from the culture broth by means of an in-situ sampling module (Flownamics Analytical Instruments Inc. Madison, USA). The sampling module is connected to the bioreactor in such a way that it is directly in contact with the

culture broth. It is an autoclavable metallic assembly provided with a ceramic membrane which selectively permits the passage of liquid medium only so that a cell free sample zone comes out of the reactor for glucose measurement.

The reliability of glucose measurements depends greatly on the stability of the FIA system. Fluid dynamics inside the FIA system plays an important role in keeping the system in a stable state. In order to keep the fluid dynamics at a steady state, the inner diameter of all the connectors used including the manifold is kept uniform as 0.8 mm. At the other end of the injection system, just before the electrode, 90 cm long Teflon tubing coiled in a zig zag pattern called the manifold is attached. This ensures a complete mixing of sample with enzyme solution. At the end of the manifold, an air trap membrane is also arranged to guarantee a bubble free sample stream to the electrode.

The buffer solution and sample solution were propelled into the FIA system using peristaltic pumps at a flow rate of 1.8 ml/min and 0.6 ml/min respectively. 35 μL of enzyme solution and 24 μL of sample were injected into the buffer stream.

The Clark electrode is integrated in acryl housing. The electrode tip is covered with a stretched semi permeable membrane separated from the electrode by an extremely thin layer of electrolyte. The membrane allows only the passage of oxygen through it and the oxygen consumed in the reaction has been measured. The overall arrangement of FIA system is shown in Figure 3.

The calibration of the system has been carried out by means of a selector, which can automatically switch in between six different standard solutions. The standard glucose solutions were prepared with concentrations ranging from 0 g/L to 1 g/L. The concentration of the glucose oxidase solution was fixed as 500000 U/L. Three measurements were taken continuously for every individual concentration and the CAFCA (Computer Assisted Flow Control and Analysis) software will automatically plot a calibration curve. The on-line measurement cycles will be performed on the basis of this calibration data. Therefore, the reliability of the measurement results will depend on the accuracy of the calibration data.

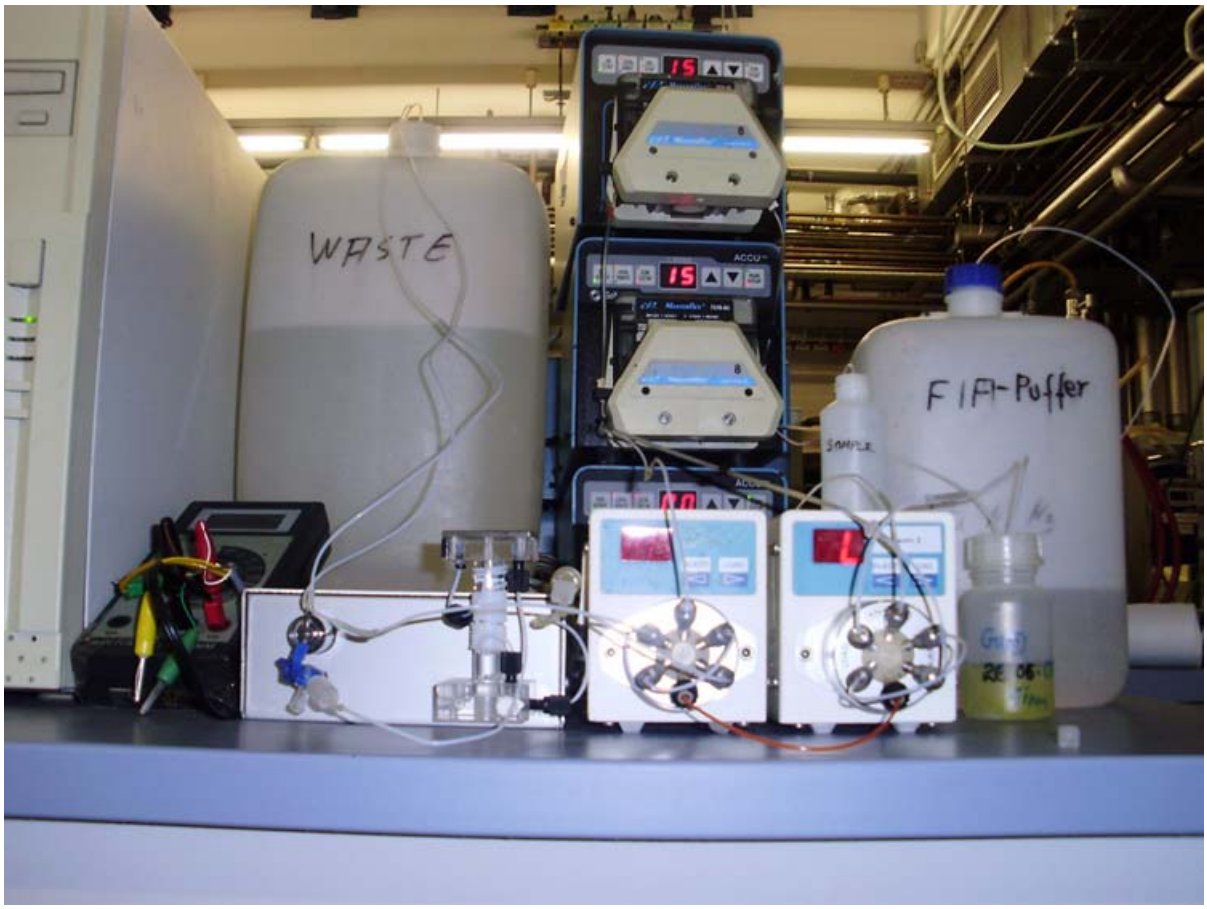

Figure 3: The FIA system

Control of the injection valves used in the FIA as well as the data acquisition and evaluation was executed by special software called CAFCA. It functions with a conventional PC with a data acquisition card PCL-818 (Adlink, Taiwan) under DOS. The programme is provided with special options such as automatic calibration, smoothening of the measurement signals as well as automatic evaluation of the measurement data. Figure 4 shows the screen of CAFCA during measurement.

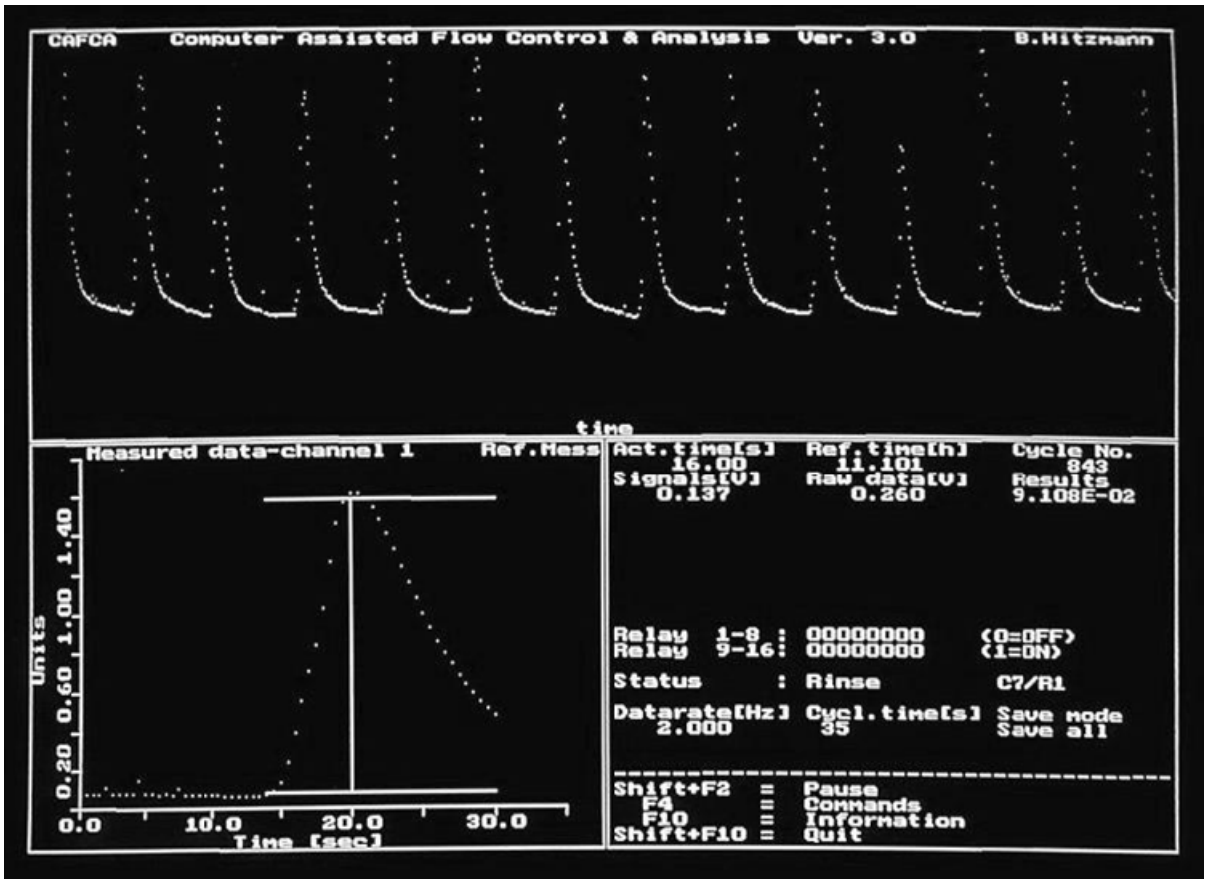

Figure 4: Screen of CAFCA during measurement.

3.2. Cultivations

The main objective of this work is to establish a control system for maximum production of biomass during the bioreactor cultivation of *E. coli* K1. A detailed description of the bioreactor used for this purpose as well as the cultivation parameters are discussed in the following sub topics.

3.2.1. The bioreactor and control unit

All the described fed-batch cultivations were performed in a jacketed stirred tank reactor made up of high quality stainless steel (working volume 2.5 L, manufactured by the mechanical workshop of Institute for Technical Chemistry, University of Hannover). It can be closed tightly with a stainless steel lid by screw arrangement. The lid has specially designed inlets for acid, base, feed solution, pre-culture, air flow etc. and outlets for off-line sampling and exhaust gas.

For the monitoring and control of the process parameters, a *BIOSTAT B*-DCU (B. Braun Biotech, Melsungen) is used. The digital control unit is used for the measurement and control

of parameters such as pH value, dissolved oxygen concentration, stirrer speed and temperature (Platinum thermometer, Pt100). The bioreactor with control unit is shown in Figure 5.

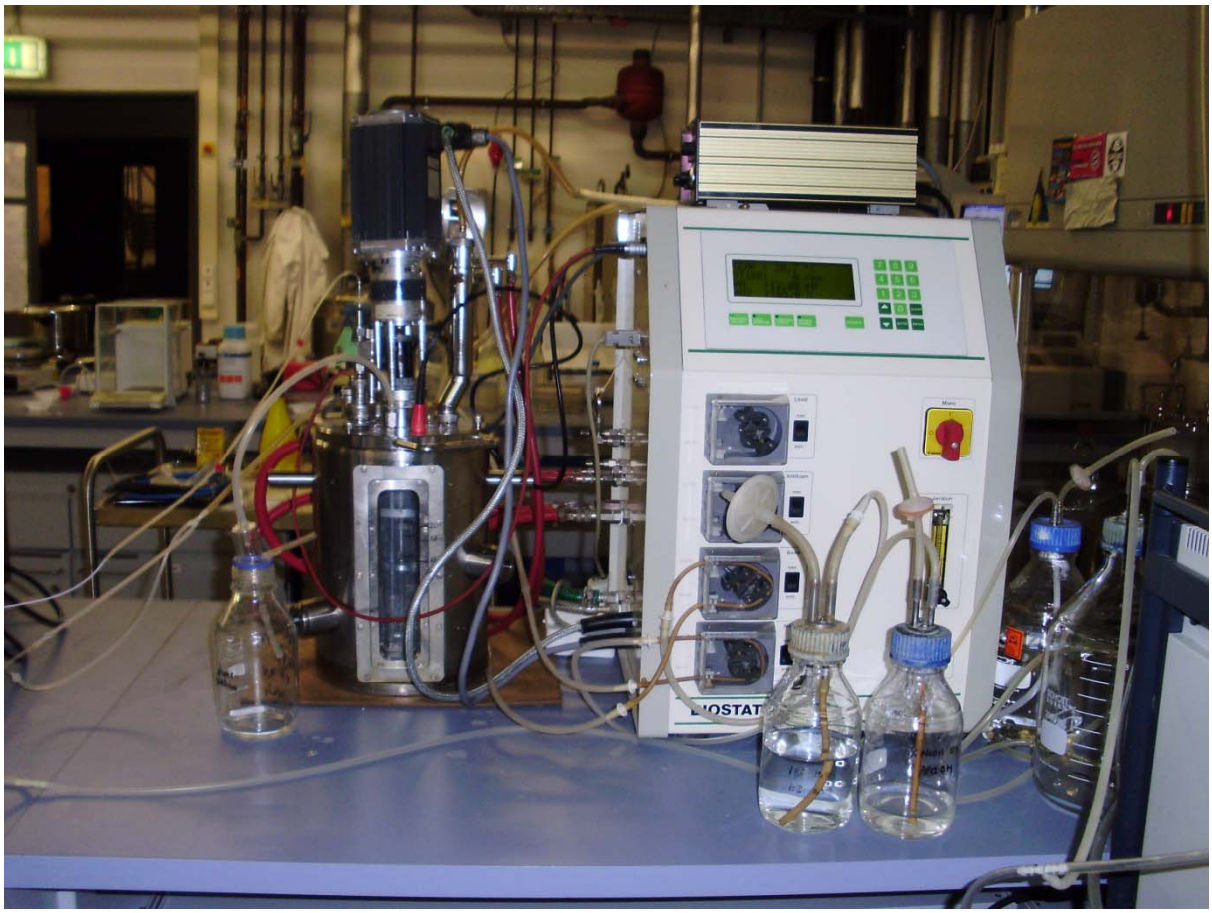

Figure 5: Bioreactor with Biostat B-DCU.

The aeration rate is controlled by a mass flow meter (Brooks mass flow meter 5851 with control unit model 5876, Brooks Instruments, Veenendaal, Holland). In order to assure the sterility of the reactor content, a sterile filter is placed at the entrance of the gas into the reactor.

A scheme of arrangement of the bioreactor with all the external equipmental arrangements is shown in Figure 6.

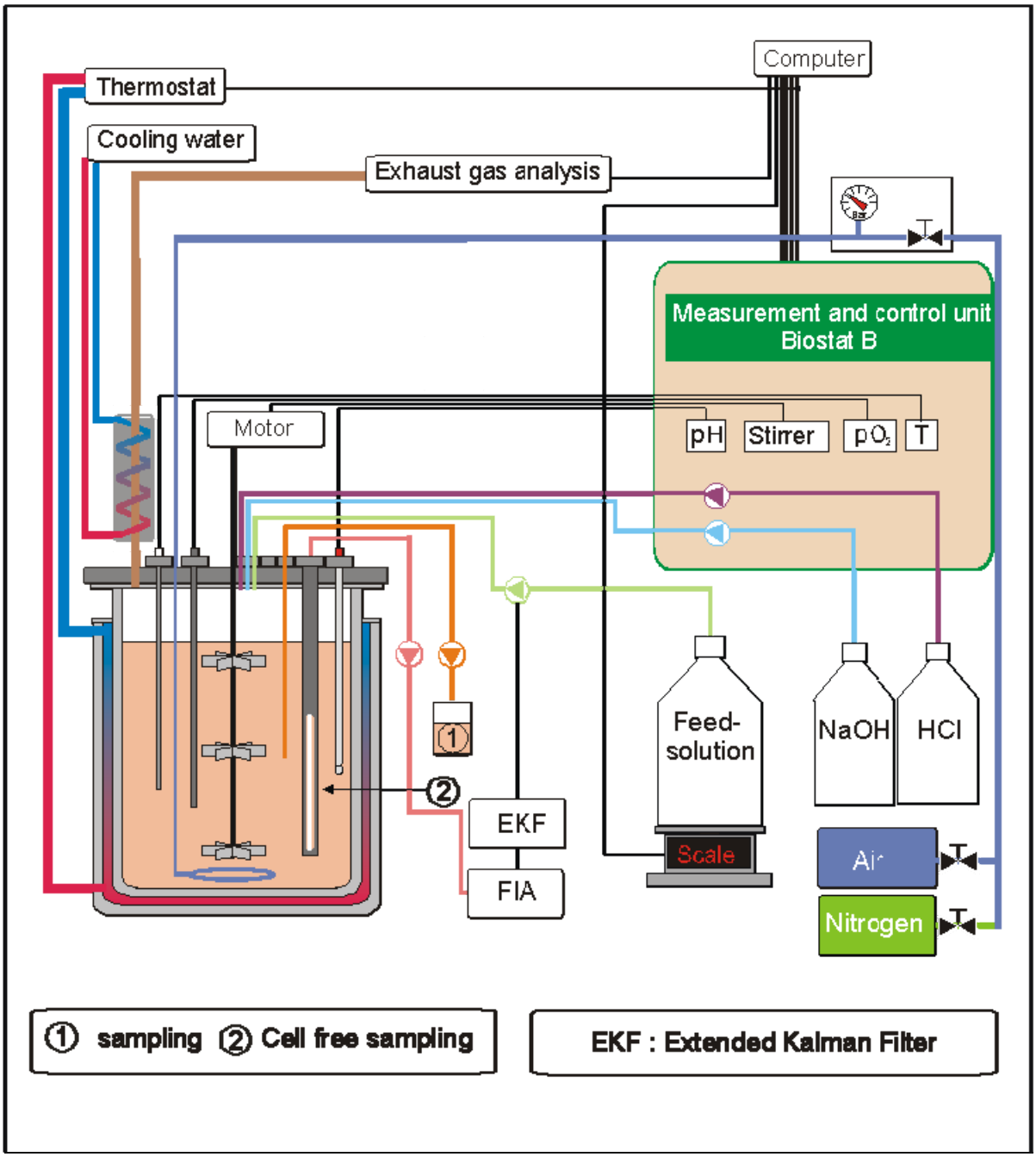

Figure 6: Scheme of set up for fermentation unit and control system.

3.2.2. Procedure for pre-culture

In all the cultivations, the wild strain *E. coli* B2032/82 serotype K1 was used. The strain is stocked in 40 % glycerol samples at -80ºC. LB medium is used as the pre-culture medium and the composition is specified in appendix A6. The LB medium is autoclaved at 120ºC for 20 minutes.

To 100 mL of the sterilised LB medium in a 500 mL conical flask, 500 µL of glycerol sample is transferred using a sterilised pipette on a clean bench and then it is transferred to an incubator and allow growing for 12-14 hours. A temperature of 37ºC and a shaking rate of 120 min^{-1} are used throughout the pre-culture process. The conical flask is provided with an outlet towards the bottom through which the inoculums is transferred to the bioreactor.

3.2.3. General parameters for cultivation process

The experiments were carried out in the reactor explained in chapter 3.2.1.The monitoring of pH value, dissolved oxygen concentration and temperature will be performed continuously. For the accurate measurement of glucose feeding into the bioreactor, the weight of the feed solution is also measured continuously. For this purpose the feed solution is placed on an electronic balance (Competence CP 8201, Satorius, Göttingen). All these on-line measurement data are stored in special software RISP (Real Time Integrating Software Platform, Institute for Technical Chemistry, University of Hannover). Off-line samples have been taken at regular intervals throughout the cultivation process. At the same time on-line measurement of glucose concentration would also be carried out by CAFCA controlled FIA system.

Before inoculation, the bioreactor would be autoclaved at 120ºC for 20 minutes, with required amount of medium inside. The glucose and buffer solution would be added after autoclaving. Usually about 100 to 200 mL of the medium will get evaporated during autoclaving. The glucose and buffer solution will be added to the bioreactor after autoclaving when the reactor temperature comes down to the required cultivation temperature. The composition of the reactor medium and the component solution are presented in appendix A3, A4 and A5. The composition of the feed solution is also mentioned in appendix A1. The composition depends on the glucose concentration. A standard composition with 100 g/L glucose concentration is given in appendix A1.

When the bioreactor parameters such as temperature, aeration rate, stirrer speed, pH etc. have been adjusted to the required values, inoculation was carried out. The general parameters used in *E. coli* K1 cultivations are comprehended in Table 1.

Table 1: General cultivation conditions used for the cultivation of *E. coli* K1.

Parameter	**Value**
Temperature	37º C
pH	7.5
Aeration rate	90 L/h
Stirrer Speed	50 to1200 rpm (cascade mode in fed-batch phase)
pH correction solutions	10 % NaOH 10 % HCl

In glucose limited fed-batch cultivations, the feeding will start after the entire batch phase. But in EKF controlled cultivations, the controller will be started before the batch phase comes to an end so that the controller will start feeding when the glucose concentration falls below the desired set point. When the batch phase is over, the stirrer will be changed to cascade mode with speed ranging from 50 rpm to 1200 rpm. Offline-samples will be collected at regular intervals.

3.3. Implementation of controllers

In glucose limited fed-batch cultivations, the substrate concentration inside the bioreactor is assumed to be zero. i.e. $\frac{dS}{dt} = 0$. Therefore from equation 17 (Page 16), the feed rate at time t can be derived as:

$$\dot{V}_F(t) = \mu_{max} \frac{S}{K_m + S} \frac{X(t)}{Y_{X/S}} \frac{1}{S_0 - S} V_R(t) \qquad [33]$$

The programme used for controlling the feeding is known as Neu-ork (Institute for Technical Chemistry, University of Hannover), executed under MS-DOS.

In EKF controlled cultivations, the controller has two components. A continuous extended Kalman filter which estimates the system state variables and smoothen the measured variable data. By assuming a suitable process model, here Monod model, and selecting the initial parameters for the Kalman filter carefully, EKF carry on the continuous prediction of the state variables and calculate the feeding rate to keep the desired substrate set point. This is a pure feed forward control. But the growth of the microorganism cannot be predicted

accurately and sometimes complex dynamics will be developed. Therefore the model based prediction alone is not enough to incorporate these unexpected changes inside the system. To compensate the inaccuracies arising from the inadequacies of the model prediction, a feedback term is applied. It contains a simple recursive PI controller. It acts as a correction to the feed forward controller. In this way, the feed forward/feedback controller can be written mathematically as:

forward /feedback controller is given which can be written as equation [33]

Pump rate = Model based control + Discrete PI control

(*Feed forward*) (*Feed back*)

$$\dot{V}_{sub}(t_i) = V(t)\frac{\mu_{max}.S(t_i).X(t_i)}{Y\left(K_m + S(t_i)\right).(S_0 - S(t_i))} + \dot{V_{PI}}(t_i) \qquad [34]$$

The volume flow rate part of the PI-controller $\dot{V_{PI}}(t_i)$ is calculated by the following equation:

$$\dot{V}_{PI}(t_i) = \dot{V}_{PI}(t_{i-1}) + q_0\left(S_{sp} - S(t_i)\right) + q_1.\left(S_{sp} - S(t_{i-1})\right) \qquad [35]$$

The value of q_0 and q_1 are taken from simulations. The Kalman Filter algorithm was implemented in Borland Delphi 4.0 (Inprise Corporation, Scotts Valley, USA) by using variables with 19 digits precision on PC running under Windows 95 (Microsoft, Redmond, USA) mode. The software runs on a separate PC with two serial ports. One port is connected to the CAFCA computer. CAFCA conveys the measured parameters to the Kalman filter. The classic filter equation was applied to estimate the current glucose concentration, whenever a new measurement was available. When the estimated value reached a point below the set point, the controller will turn on automatically. The integration of the differential equation has been performed by a 4th order Runge-Kutta method. The second serial port is connected to the pump (ACCU CP10, SciLog, USA with pump head Masterflex Coleparmer Instrument Co., Illinois) which is used for feeding glucose into the bioreactor. The flow rate of the glucose solution into the bioreactor is continuously calculated. The pump will start with an initial pump rate of 5 %. If the mean value of the calculated feed rate is below 5 %, then the pump will always fluctuate in between 0 % and 5 %. The PI controller values were kept constant in all the discussed cultivations in this work. The values used are q_0 = 1.4 L^2/gh and q_1 = -1.1 L^2/gh. Depending on the desired set point concentration of glucose, different values

were selected for *R* and those values were specified while discussing every cultivation separately. For all the cultivations, the diagonal values of P and *Q* are tabulated in Table 2.

Table 2: *P* and *Q* values during the cultivation of *E. coli* K1

		Initial Values of *P*	
Q (1,1)	0.001 g^2/L^2h	*P* (1,1)	0.1 g^2/L^2
Q (2,2)	0.001 g^2/L^2h	*P* (2,2)	0.02 g^2/L^2
Q (3,3)	0.05 L/h^3	*P* (3,3)	0.2 $1/h^2$
Q (4,4)	0 L^2/h	*P* (4,4)	0 L^2

P (i, j) = 0 for i≠ j

3.4. The process analysis

3.4.1. On-line analysis

3.4.1.1. Measurement of pH values

For the measurement of pH value, a sterrilisable (autoclaveable) pH electrode (Polyclave 225, Hamilton Bonaduz AG, Bonaduz, Schweitz) is used. It is build up with a glass and reference electrode. The voltage in between the electrodes is used as the measurement variable, which is measured by a voltmeter.

The calibration of the electrode has been carried out before autoclaving using standard buffers of pH 4.01 and 9.5.

3.4.1.2. Measurement of dissolved oxygen concentration

The dissolved oxygen concentration is measured as oxygen partial pressure by means of an autoclavable electrode (inpro 6000, Mettler Toledo, Giessen). The measurement principle is Clark's principle.

After autoclaving, the electrode should be left at least for 6 hours to get polarised and then calibrated. The calibration has been done by passing nitrogen gas through the medium (Linde Gas AG, Höllriegelskreuth). The dissolved oxygen concentration will then sink to a value of 0 %. Then, the oxygen concentration might be brought back to the saturation value (100 %) by passing air through the bioreactor under actual cultivation conditions (Stirrer speed, temperature, pH and aeration rate).

3.4.1.3. Exhaust gas analysis

The exhaust gas analysis is carried out by S710 gas analyser (SICK MAIHAK GmbH, Reute, Germany). It is provided with two analysis modules; OXOR-P and UNOR.

UNOR measures the CO_2 concentration. It works on the principle of NDIR (Non Dispersive Infrared Absorption) with a pneumatic detector. The measurement is based on the resonance absorption from vibrational rotational bands in the middle infra red region (800-3000 nm).

The oxygen concentration is measured by the analysis module OXOR-P which is based on the paramagnetic property of the oxygen molecule. This method has been extensively dealt by Hüll (Hüll 2007).

3. 4. 2. Off-line analysis

In all the cultivations off-line samples were taken at regular intervals. The samples were collected in a cooled sampling tube so that the metabolism of the organism can be inactivated immediately. In high cell density conditions it is very important because otherwise the off-line measurements do not make any sense.

3.4. 2.1. Optical density

The optical density of the culture broth has been measured throughout the cultivation process in a spectrophotometer (Unikon 922, Beckmann, USA) at a wavelength of 580 nm. At higher cell densities, the culture broth should be appropriately diluted for reliable measurement data.

3.4.2.2. Biomass concentration

The biomass concentration is measured in gravimetric method. 2 mL of the culture broth is taken in a micro reaction tube (Eppendorf, Hamburg). The micro tubes must be pre-heated in an oven at 110ºC for 24 hours and pre-weighed. This is to ensure the complete dryness of the tubes. The tubes with culture broth are then centrifuged to get the biomass sediment (3 min, 13,000 rpm, Centrifuge 5415 D, Eppendorf, Hamburg, Deutschland). The micro tubes containing the pellets are again transferred to the oven and heated for 24 hours at a temperature of 110ºC. Then allowed to cool in desiccators and then weighed. The difference in the weights gives the biomass concentration.

3.4.2.3. Glucose concentration

The off-line measurement of glucose concentration has been carried out by YSI 2700 Analyser (Yellow Spring Instruments, England). The method is based on the principle of enzymatic reaction of glucose with GOD which has already been explained in chapter 2.1 (Page 3).

3.4.2.4. Quantitative detection of acetate

In all the cultivations, quantitative detection of acetate has been carried out by gas chromatographic method (Shimadzu, Duizburg, Germany). N-propane is used as the internal standard.

3.4.2.5. Polysialic acid determination

Colorimetric method is used for the quantitative determination of polySia during the cultivation process. It is a modified version of the thiobarbituric acid method described by Warren (Warren 1959) and Aminoff (Aminoff 1961). 50 µL of the homogenized sample was first hydrolysed by the addition of 100 µL of 0.1M H_3PO_4. Added 100 µL of distilled water into this mixture and kept for hydrolysis at 70º C for 18 hours. After hydrolysis, the sample was neutralised by adding 100 µL of 0.1 M NaOH. The neutralised samples will be oxidised and reduced successively in the following steps. The oxidation was carried out by the addition of 100 µL periodic acid (0.2 M) in 0.5 % [VV^{-1}] H_3PO_4. Then it was kept at 37º C for 30 minutes. On completion of 30 minutes of oxidation, the sample was subjected to reduction by adding 500 µL $NaAsO_2$-solution (0.38 M) in 2 % [VV^{-1}] H_2SO_4. Then 500 µL thiobarbituric acid solution (0.2 M) in 1.2 % [VV^{-1}] NaOH was added into it followed by incubation for 13 minutes at 95º C. The processed samples will then be suddenly cooled in a water bath. It will have a pink colour and this will be extracted with cyclohexanone and the extinction was measured at 549 nm.

3.5. Carbon Balance

A carbon balance can be made by the accurate measurement of the carbon flux that is going in and coming out. Carbon going in is calculated as the sum of the carbon that is going in to the reactor in the form of glucose inside the medium, biomass and acetate in the beginning of

the batch cultivation phase as well as the carbon in the feeding solution during the feeding phase.

$$C_{in} = C_{medium} + C_{Biomass} + C_{Acetate} + C_{Feed} \quad [36]$$

Carbon coming out is calculated as the sum of carbon in the form of glucose inside the medium, biomass and acetate as well as the carbon in the exhaust gas. Carbon that is taken out as biomass and acetate during offline sampling also must be added to this.

$$C_{out} = C_{medium} + C_{Biomass} + C_{Acetate} + C_{Exhaust\ gas} + C_{Offline\ samples}. \quad [37]$$

The ratio of C_{out} to C_{in} gives the carbon recovery index during the cultivation.

From these data, the carbon flux coming out as biomass, carbon dioxide and acetate is calculated with respect to the carbon that is going in.

4. Results and discussions

The shaker flask conditions for the cultivation of *E. coli* K1 were scaled up to 1.5 L for batch cultivations. Batch cultivations with different initial glucose concentrations were conducted and evaluated. In order to study the effect of growth rate on the production of biomass and polysialic acid, fed-batch cultivations were carried out under glucose limited conditions at a constant growth rate with exponential feeding strategy. Caution was taken to see that the growth rate was kept at lower values. Since this fed-batch tactic lack the feedback control, another set of fed-batch cultivations were performed with feed forward/feedback controller. The basic assumption of these cultivations was to control the glucose concentration of the culture broth at a desired set point. This control system is established by a model based substrate controller based on FIA measurements. Successfully configured extended Kalman filter was used for feed forward/feedback control. Comparative evaluations of batch, glucose limited fed-batch with exponential feeding as well as fed-batch with EKF control are presented in the first section of this topic. EKF controlled cultivations at different glucose set points were discussed in detail in the second section with a focus on the problems encountered during the cultivation process.

4.1. Comparison of biomass and PSA production in different types of cultivations

Different types of cultivations have been carried out to evaluate the biomass and PSA production. Batch cultivations, fed-batch cultivations with exponential feeding and EKF controlled cultivations are discussed under this part of the thesis.

4.1.1. Batch cultivations

Batch cultivations of *E.coli* K1 were performed as an initial step for studying the production process of biomass and polysialic acid. Two cultivations with a higher and lower initial glucose concentrations are discussed here. Both the cultivations were carried out under identical conditions in a 2.5 L bioreactor with a constant aeration rate of 90 L/h and a stirrer speed of 800 rpm at 37ºC. The adapted procedure is the same as discussed in chapter 3.2 (Page 28). These data were used for process modelling.

4.1.1.1 Cultivation 1

In order to study the cultivation process of *E.coli* K1 and the production of biomass and polysialic acid, batch cultivation with a higher glucose concentration was carried out. In this

cultivation, the initial glucose concentration was 19 g/L. The experimental set up was the same as described in chapter 3.2 (Page 28). The medium composition is depicted in appendix A4. An overview of the carbon production rate and oxygen uptake rate during the cultivation is illustrated in Figure 7.

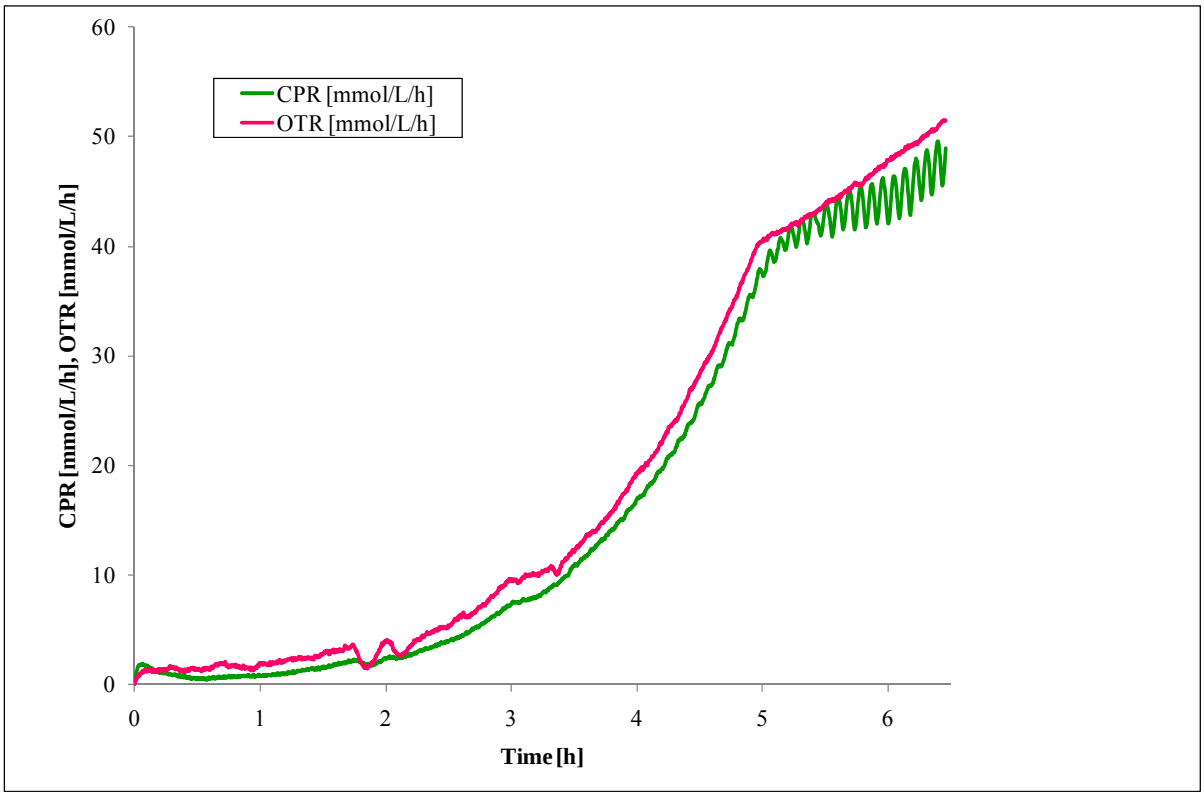

Figure 7: CPR and OTR during the batch cultivation of *E. coli* K1 with 19 g/L initial glucose concentration.

It is evident from Figure 7 that till 4.9 h, the bacterial growth was uninterrupted. But at this particular point of time, the CPR and OTR curve deviate from the normal path and was following another pattern of increase. This may be due to the oxygen limitation inside the bioreactor. The pO_2 curve (Figure 8) shows that at this point of time the dissolved oxygen reached a value of 0 %. But still there was about 12 g/L glucose inside. The maximum amount of acetate found during the cultivation was 1.74 g/L which is observed at 6.6 h. The glucose and acetate concentration are plotted in Figure 9.

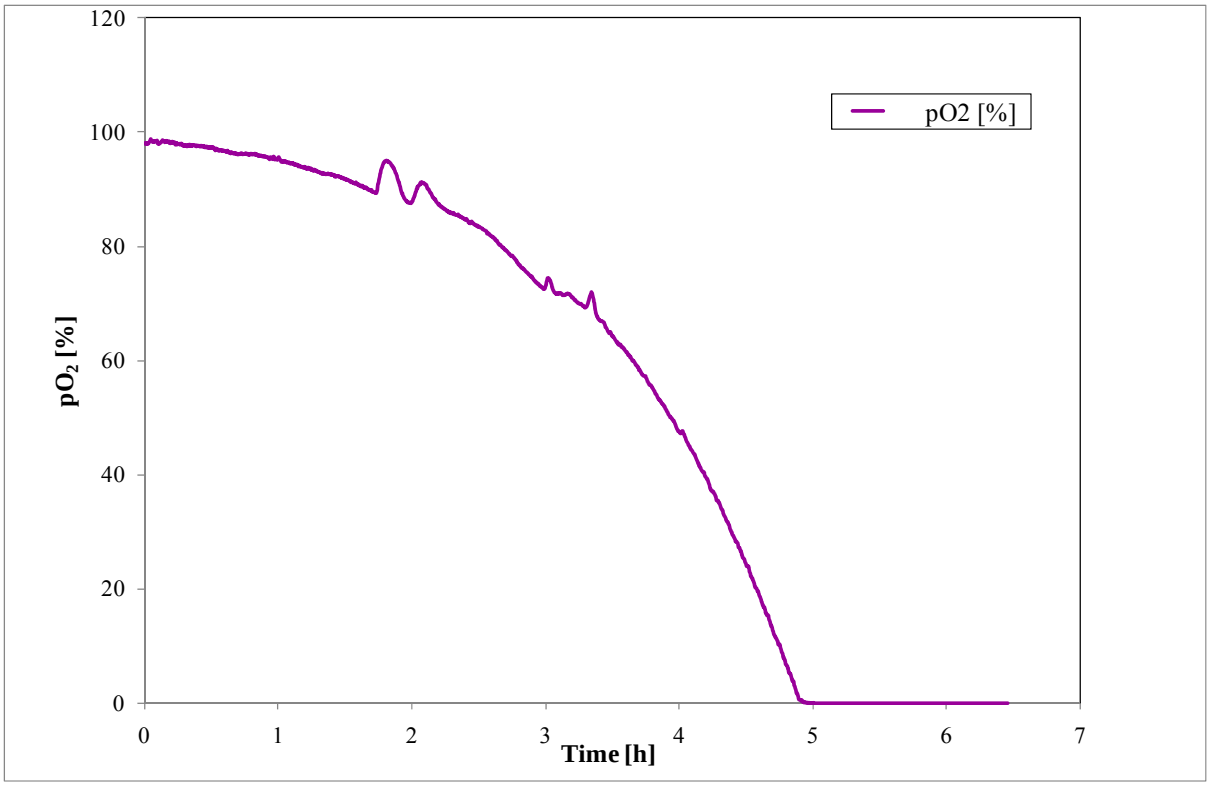

Figure 8: The dissolved oxygen concentration during the batch cultivation of *E. coli* K1 with initial glucose concentration of 19 g/L.

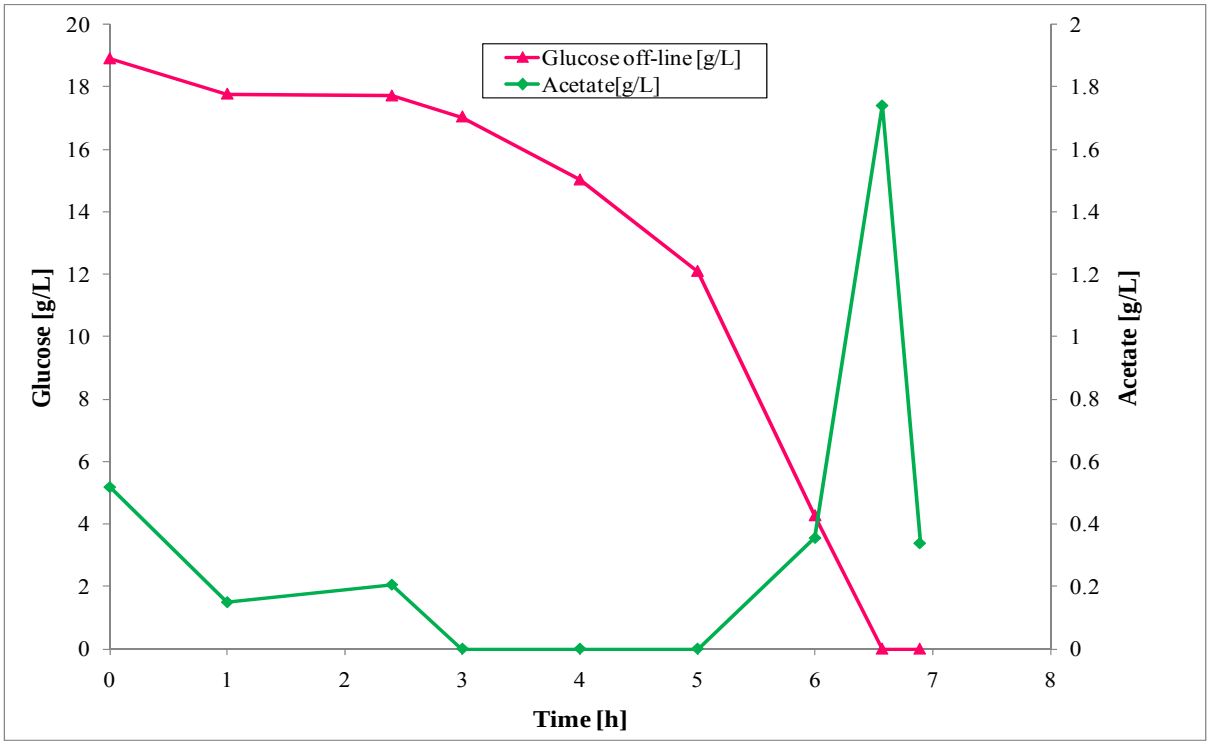

Figure 9: Glucose and acetate concentration during the batch cultivation of *E. coli* K1 with an initial glucose concentration of 19 g/L.

It is observable that the acetate concentration reached its maximum value when the glucose concentration reached zero. But after about half an hour, the acetate concentration has got reduced to 0.33 g/L. This indicates that the bacteria consumed acetate in the absence of the substrate, glucose.

As one can notice in Figure 10 the biomass concentration reached a value of 6.3 g/L at the end of the cultivation. The PSA amount reached a point of 0.5 g/L in the batch cultivation. The increase of biomass and PSA concentration in Figure 10 seems to be proportional.

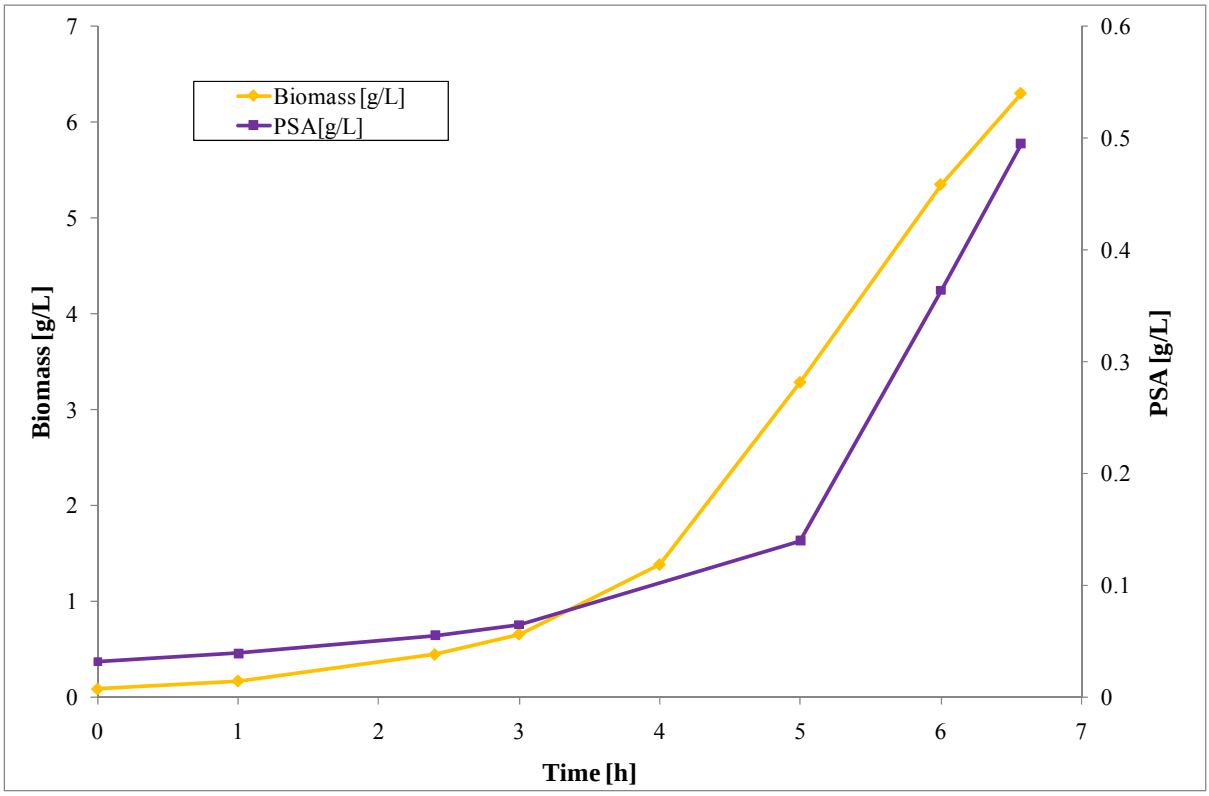

Figure 10: The biomass and PSA concentration during the batch cultivation of *E. coli* K1 with initial glucose concentration 19 g/L.

A graph is plotted (Figure 11) with biomass against PSA and the intercept and slope has been found out to be 0.0081 g/L and 0.068 $g_{PSA}/g_{Biomass}$ respectively.

Thus the batch cultivation with 19 g/L initial glucose concentration gives a biomass yield of 0.36 g/g and PSA yield of 0.019 g/g. These values were calculated using the theoretical values of biomass and PSA concentrations.

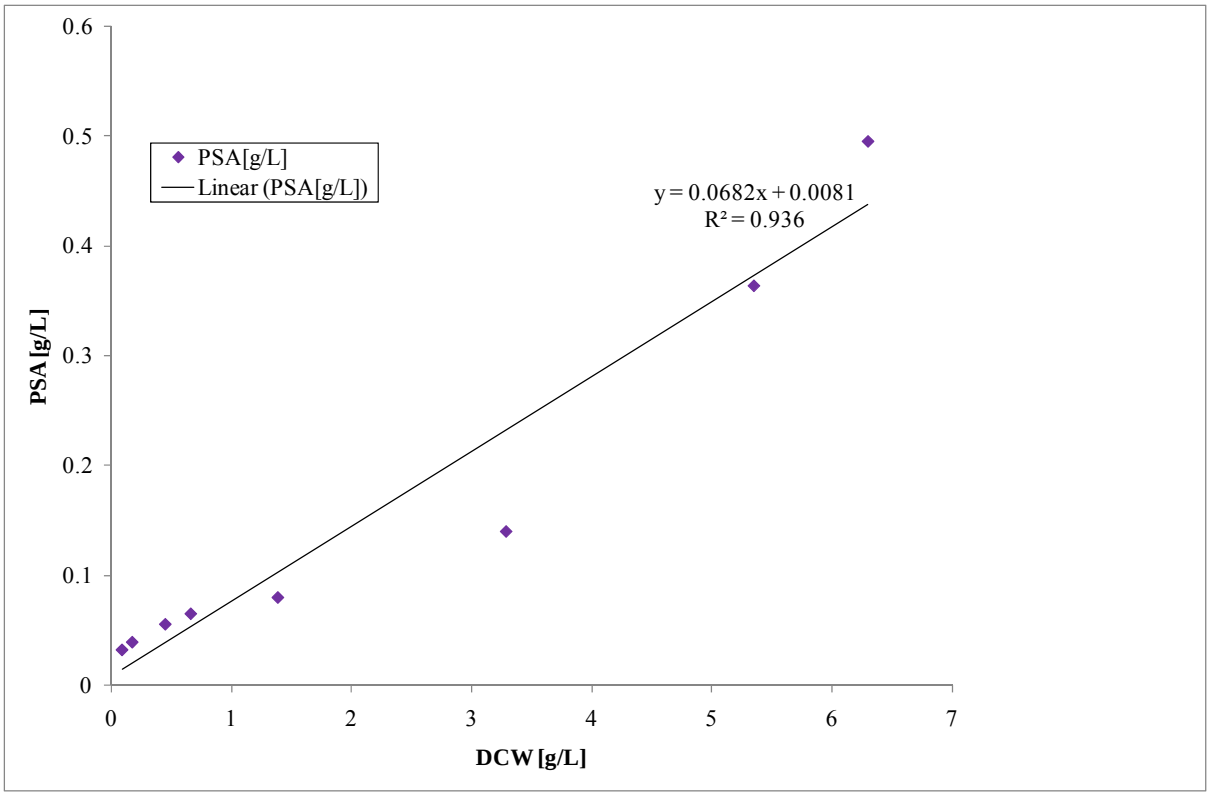

Figure 11: Plot of biomass against PSA in a batch cultivation with 19 g/L initial glucose concentration.

4.1.1.2. Cultivation 2

To investigate the PSA production without oxygen limitation batch cultivation with initial glucose concentration of 5 g/L was carried out. The medium composition and experimental set up were kept as the same as that followed in cultivation 1. The overall performance of the cultivation is illustrated in Figures 12 as CPR and OTR. Since the initial glucose concentration is comparably lower, no oxygen limitation or acetate accumulation was observed in this cultivation. CPR and OTR are increasing exponentially throughout the cultivation which indicates that the bacterial growth is unlimited in this batch cultivation.

The maximum amount of acetate in the cultivation was 0.56 g/L which is observed at the beginning of the cultivation. This amount is comparable to the initial acetate concentration in cultivation 1 and came from the pre-culture. The glucose concentration and acetate concentration are plotted in Figure 13. From the second hour of cultivation no detectable amount of acetate has been observed in the culture broth.

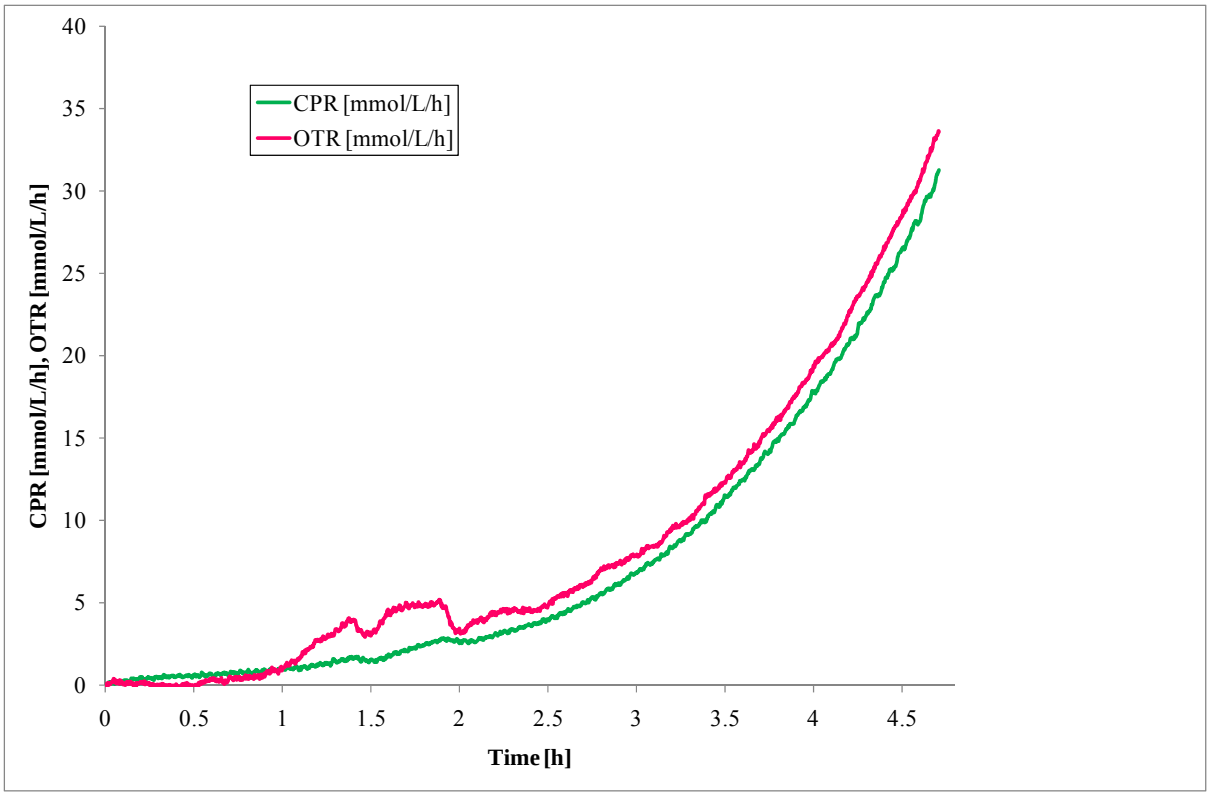

Figure 12: The trend of CPR and OTR during the batch cultivation of *E. coli* K1 with 5 g/L initial glucose concentration.

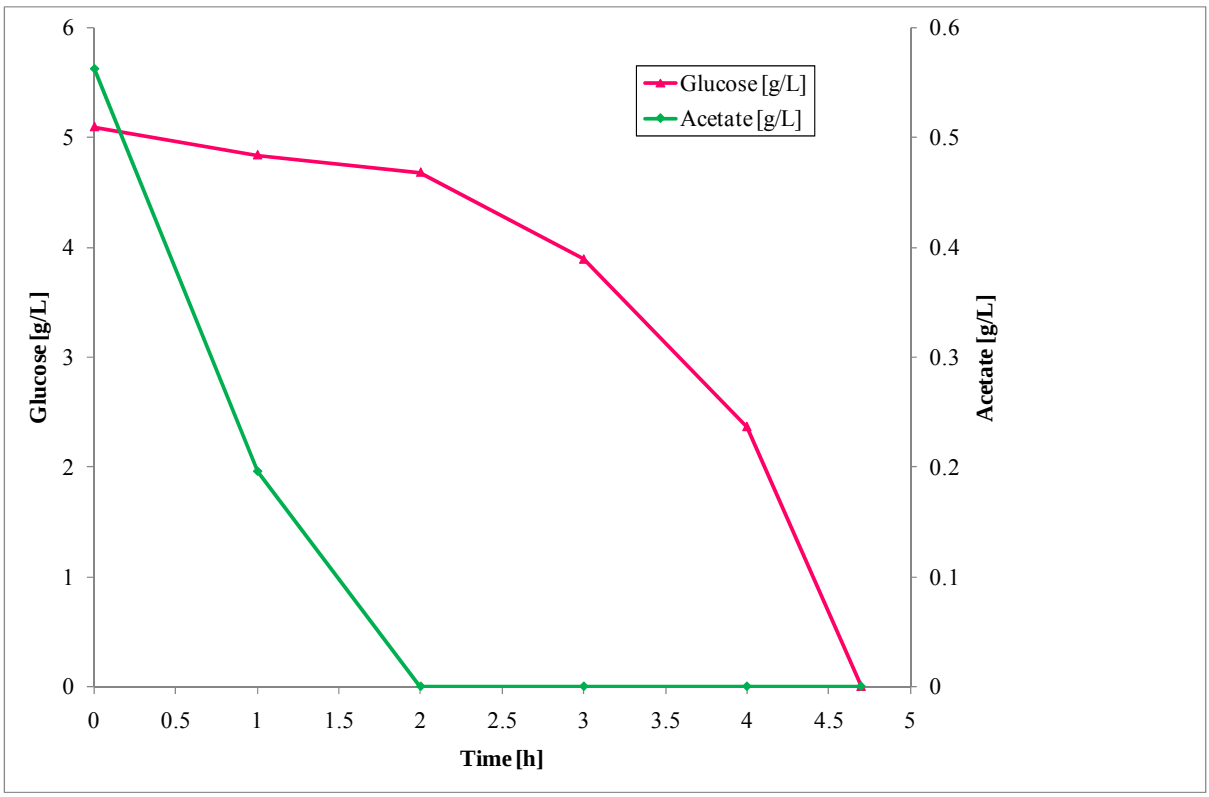

Figure 13: Glucose and acetate concentration during the batch cultivation of *E. coli* K1 with initial glucose concentration 5 g/L.

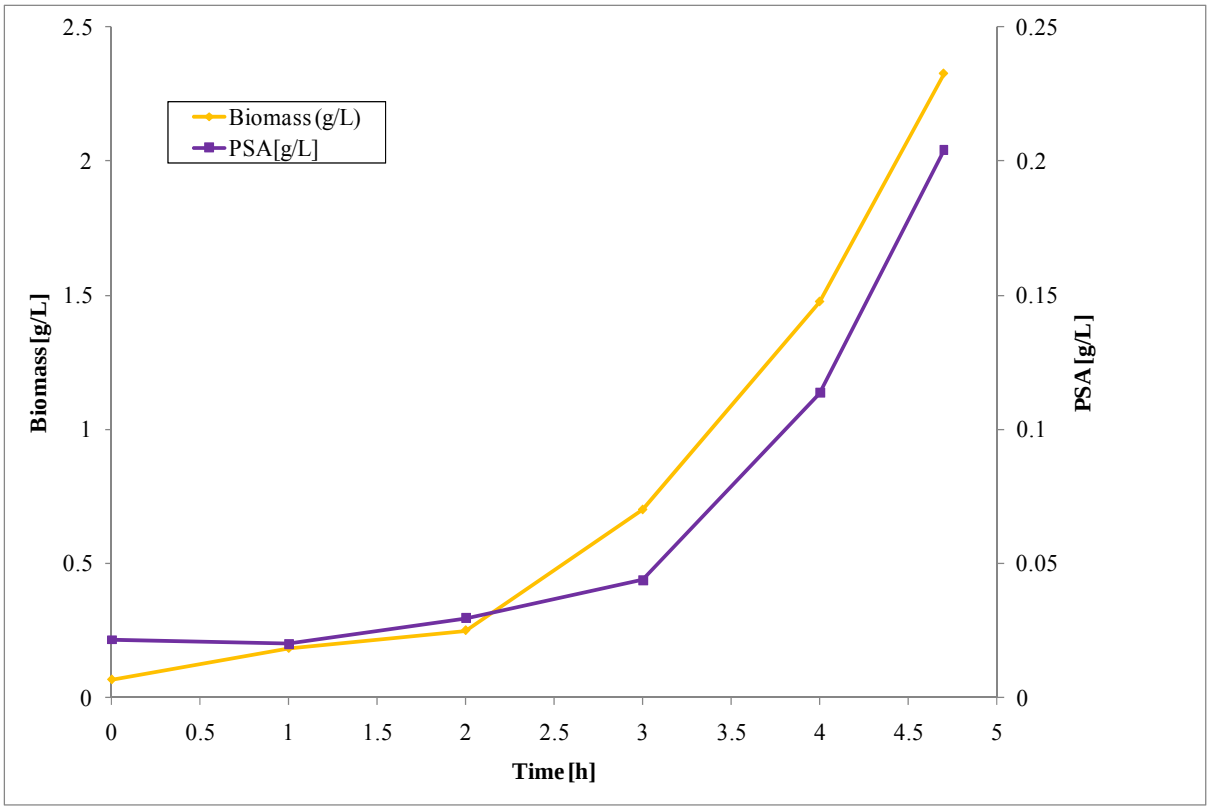

Figure 14: Biomass and acetate concentration during the cultivation *of E. coli* K1 with initial glucose concentration 5 g/L.

As depicted in Figure 14, the biomass reached a value of 2.33 g/L and the PSA amount was 0.20 g/L towards the end of the batch process. It resulted in a biomass yield of 0.46 g/g and PSA yield of 0.027 g/g when calculated using the theoretical values of biomass and PSA concentrations during the cultivation. Thus the used up glucose is converted effectively to biomass in this cultivation since the relative amount of acetate is lower compared to cultivation 1.

In Figure 15, biomass is plotted against PSA to find out the intercept and slope and it is found that the intercept is 0.0046 g/L and the slope is 0.081 $g_{PSA}/g_{Biomass}$.

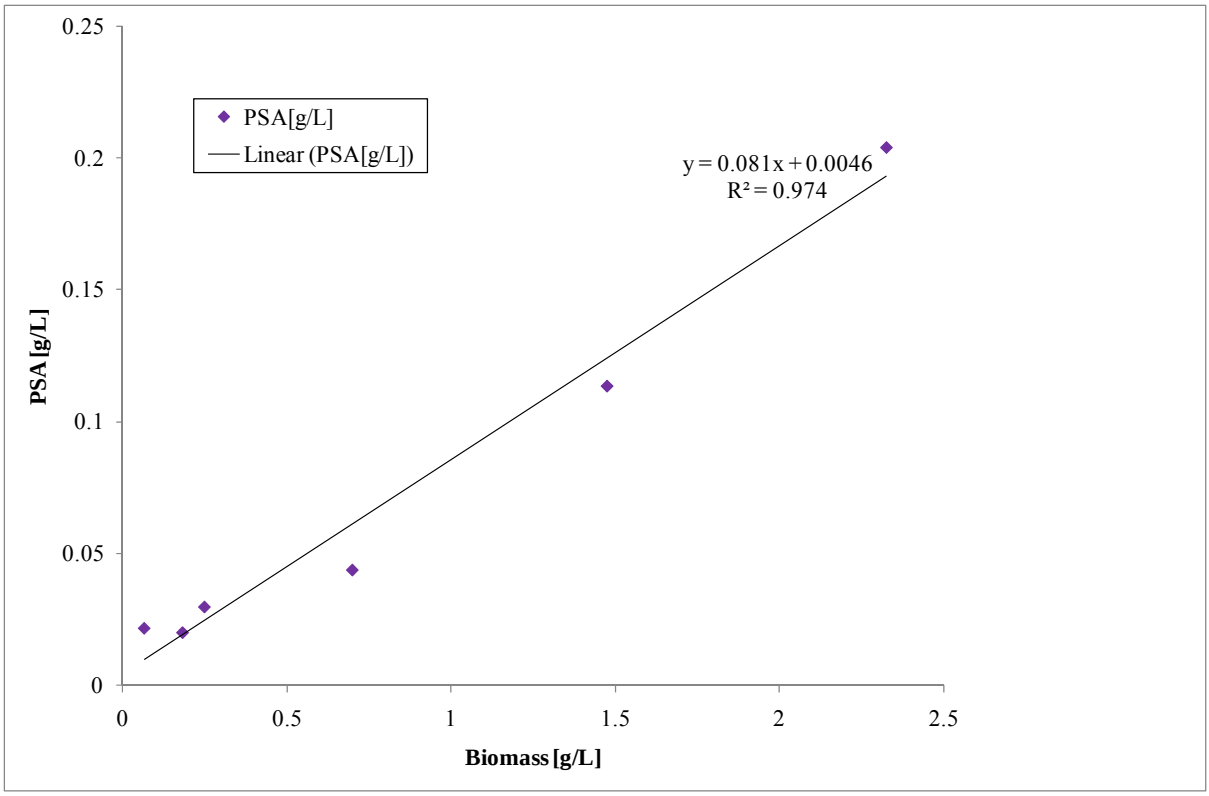

Figure 15: Plot of biomass against PSA in the batch cultivation of *E. coli* K1 with 5 g/L initial glucose concentration.

4.1.1. 3. Comparison of the batch cultivations

In the discussed batch cultivations, the glucose concentrations were selected to be with a great difference to study the effect of high glucose concentration on the overall performance of the cultivation process. As expected, with high glucose concentration, the probability of oxygen limitation is high. The overall outcomes of the two cultivations are comprehended in Table 3.

The results that are recorded in Table 3 show that the yield factor of both biomass and PSA increases when the initial glucose concentration is very low. The biomass yield increased from 0.37 g/g to 0.46 g/g when the initial glucose concentration is decreased from 19 g/L to 5 g/L. The PSA yield also increased from 0.019 g/g to 0.027 g/g.

Table 3: Comparison of output from the two different batch cultivations

Parameter	**Cultivation 1 (initial glucose 19 g/L)**	**Cultivation 2 (Initial glucose 5 g/L)**
Cultivation Duration	6.45 h	4.7 h
Maximum OD	21.71	9.23
Biomass Yield	0.37 g/g	0.46 g/g
PSA Yield	0.019 g/g	0.027 g/g
Maximum Acetate	1.74 g/L	0.56 g/L
Growth rate	0.67 h^{-1}	0.75 h^{-1}

The lower biomass as well as PSA yield during cultivation 1 might be due to the oxygen limitation inside the bioreactor at 4.9 h. The yield factor calculated till this particular point is comparably higher. The biomass yield was 0.43 g/g whereas the PSA yield was 0.037 g/g. But after 4.9 h of cultivation, when exposed to oxygen limitation, *E. coli* shifts to fermentative metabolism. As a result of fermentative metabolism oxidised fermentation product, acetate has got released into the cultivation media. In cultivation 1, the acetate concentration increased from a value of 0 g/L to 1.74 g/L within 1.57 hours after oxygen limitation. From this time point the growth rate also decreased from 0.71 h^{-1} to 0.65 h^{-1} since the carbon flux started to get diverted to acetate production. When the initial glucose concentration is less, acetate inhibition or oxygen limitation might not be possible. Hence a higher yield is obtained. CPR and OTR are also decreasing at this point of time.

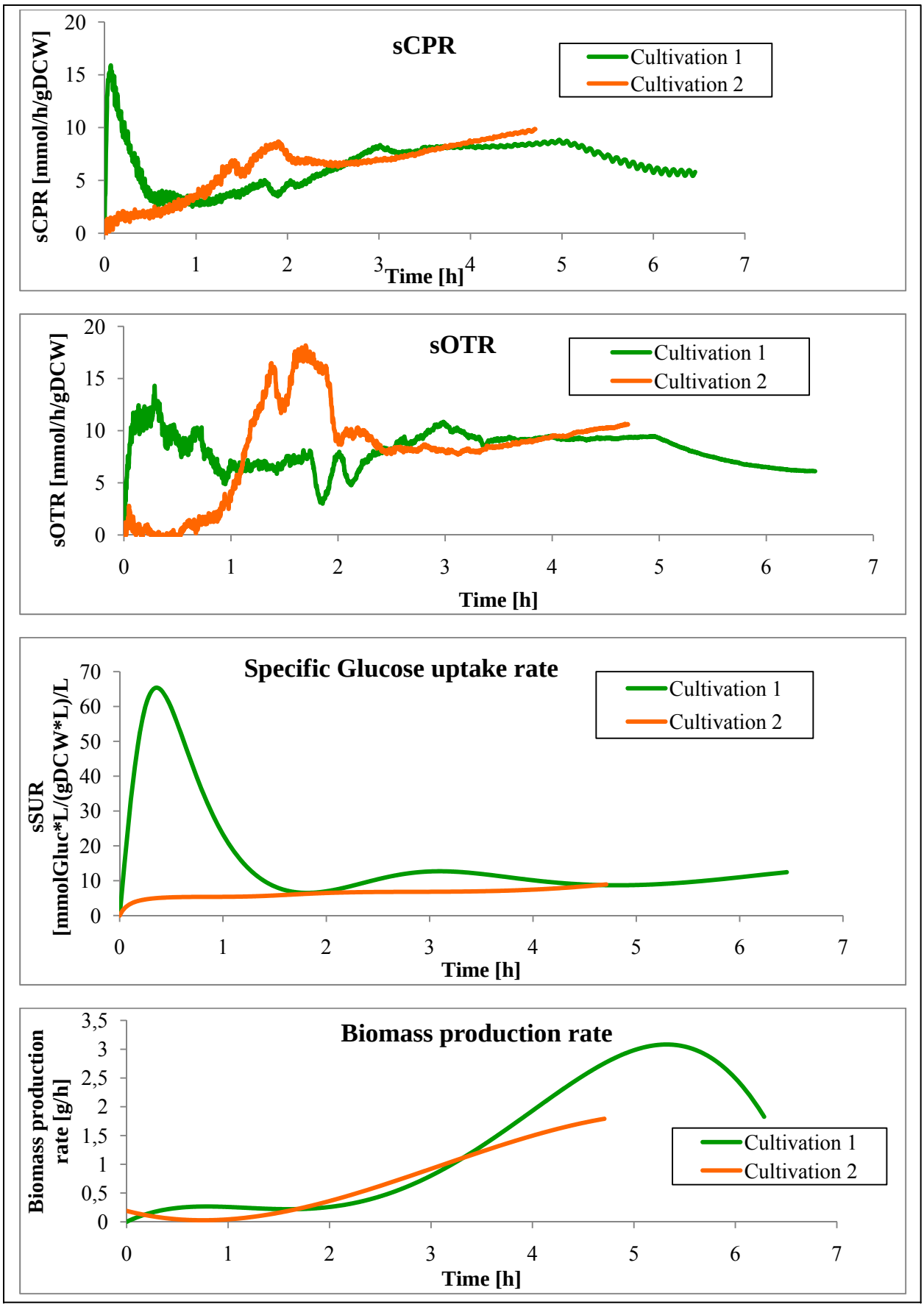

Figure 16: Comparison of the specific CPR, OTR and glucose uptake rate as well as biomass production rate during batch cultivations of *E. coli* K1.

Figure 16 illustrates the specific uptake and production rates during the batch cultivation of *E. coli* K1. As one could see in Figure 16, OTR, CPR and biomass production rate are decreasing in cultivation 1 after 4.9 hours, the point at which oxygen limitation starts. This also signifies the conversion of the bacterial metabolism from oxidative to fermentative. But the glucose uptake rate does not show any considerable change. It might be possible that the substrate taken in is converted to acetate during this period rather than to biomass.

In Figure 16, one could realise that in the beginning of the batch phase, the CPR and OTR are changing unsteadily for a few hours in the beginning. This might be the effect of LB medium used for the pre-culture. The effect of this complex medium exists for a few more hours after getting transferred to the defined medium. This effect will have a stronger influence on the process when the duration of the cultivation is shorter. This might be a reason for higher yield coefficient in cultivation 2. As illustrated in Figure 10 and Figure 14, in the beginning of the cultivation the coefficient of PSA to biomass is higher and later it started to decrease. This might also be the influence of LB medium. In complex media, the bacteria can grow well and also can integrate more PSA into the cell wall.

The efficiency of the two discussed cultivations can be evaluated by making a carbon balance. For making carbon balance, the elemental composition of biomass has been determined and an average value of 42.7 % is used for the calculations. The data from carbon balance of the two batch cultivations are summarized in Table 4. In cultivation 1, the data from 0 to 5 hours has been considered for the carbon balance. In cultivation 2, data from 0 to 4 hours were considered.

Table 4: Carbon balance data of the two batch cultivations.

Cultivation	Carbon content of the biomass [%]	Carbon taken in [g]	Carbon coming out [g]	Ratio [C_{out}/C_{in}]
C1	42.7	11.71	10.14	0.87
C2		3.44	2.78	0.81

The distribution of the carbon flux is given in Table 5. The data is expressed as the percentage of carbon. The carbon distribution in cultivation 1, calculated till 6 hours (after oxygen limitation) is represented as C1* in Table 5.

Table 5: Carbon flux distribution in batch cultivations of *E. coli* K1.

Cultivation No	Biomass [%]	CO_2 [%]	Acetate [%]
C1	65.57	34.43	0
C1*	65.05	31.29	3.6
C2	67.06	32.94	0

For comparison, the carbon balance calculated till 6th hour (C1*) is represented in figure 17. A part of carbon flux is getting diverted to acetate also in cultivation 1 with higher glucose concentration. That might be a reason for a reduction in biomass yield during this cultivation.

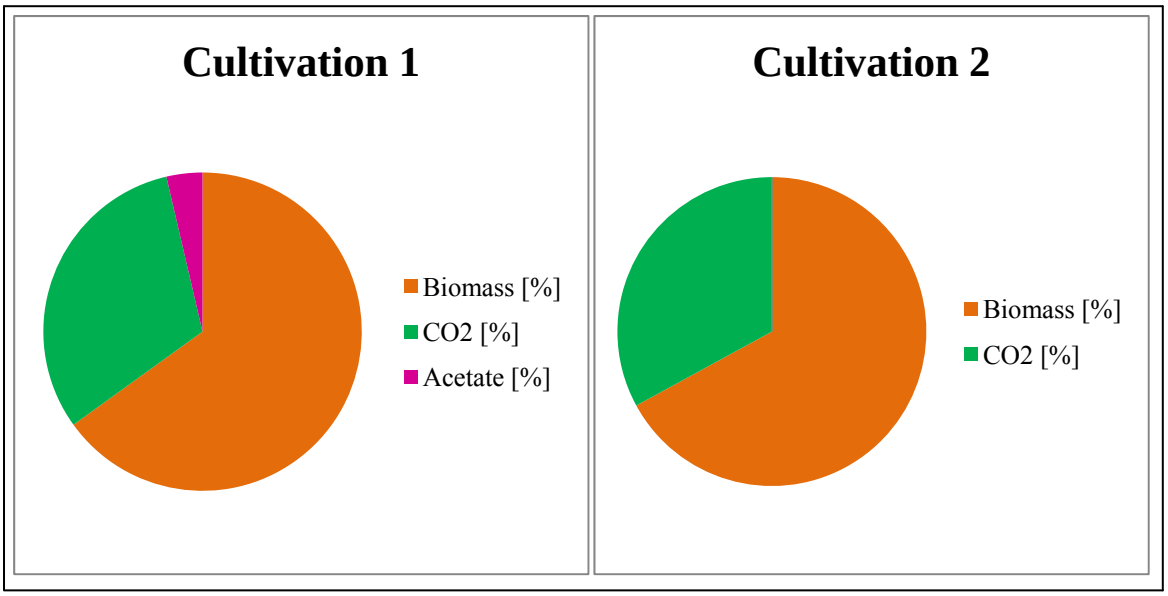

Figure 17: Carbon distribution during batch cultivations of *E. coli* K1.

From the overall evaluation of the batch cultivations, it is evident that the yield coefficient of both biomass and PSA will be higher when the initial glucose concentration is lower. But it is not interesting in industrial point of view. The rate of formation of the product also must be taken care in such purposes. But the rate of formation of PSA is 0.048 g/h in cultivation 1 and 0.029 g/h in cultivation 2. Therefore, cultivation with lower initial glucose concentration might not be acceptable as a suitable production method for PSA even when the yield is higher compared to the one with higher initial glucose concentration.

The result of cultivation 1 can be compared with similar data in the literature (Rode 2008). According to Rode et al. with the consumption of 26.6 g of glucose a PSA yield of 0.020 g/g is obtained, which is almost similar to the value obtained in cultivation 1. Here, with the consumption of 28.5 g of glucose a PSA yield of 0.019 g/g is obtained which is comparable.

4.1.2. Cultivations with exponential feeding

Besides batch cultivations, fed-batch cultivations are analysed with respect to PSA and biomass production. A set of experiments were performed with an exponential feeding strategy. In this type of cultivations, it is assumed that the bacteria are growing at a fixed specified growth rate. The feeding was carried out with such an assumption that the biomass inside the reactor was increasing according to the specified growth rate and the glucose concentration inside the bioreactor was arbitrarily zero. Even if so many experiments were performed with this strategy, three sets of results are discussed here in the following sub topics. The initial values of the parameters given to the software are summarised in Table 6.

Table 6: Initial values given to the exponential feeding software during cultivation of *E. coli*-K1 at different growth rates

Parameter	Cultivation 3	Cultivation 4	Cultivation 5
Growth Rate	0.15 h^{-1}	0.25 h^{-1}	0.35 h^{-1}
Biomass	2.5 g/L	2.63 g/L	3.6 g/L
Volume	1.45 L	1.45 L	1.47 g/L
Yield	0.5 g/g	0.39 g/g	0.5 g/g
Glucose	0.04 g/L	0.04 g/L	0.04 g/L

The fed-batch cultivations with exponential feeding were carried out by a special software programme called Neu-ORK (Ordinary Runge-Kutta) programme. The programme uses equation number 16 to 18, but not with the Monod model, but with ordinary exponential growth model.

4.1.2.1. Cultivation 3

In this chapter, a glucose limited fed-batch cultivation of *E. coli* K1 is described which had been run at a growth rate of 0.15 h^{-1}. The medium composition is presented in Appendix A4. In this cultivation, the initial batch phase glucose concentration was 5 g/L. The batch phase ended at 4.3 h and the feeding continued till 16.5 hours. Even though the growth rate value given to the software was 0.15 h^{-1}, the off-line calculation gives a growth rate of 0.11 h^{-1}. The exhaust gas analysis shows that the bacteria were growing well in glucose limited conditions even when the growth rate was having such a lower value. The CPR and OTR values, increasing continuously till the end of the cultivation (Figure 18). The data only up to 16.3 h of cultivation is discussed under this chapter.

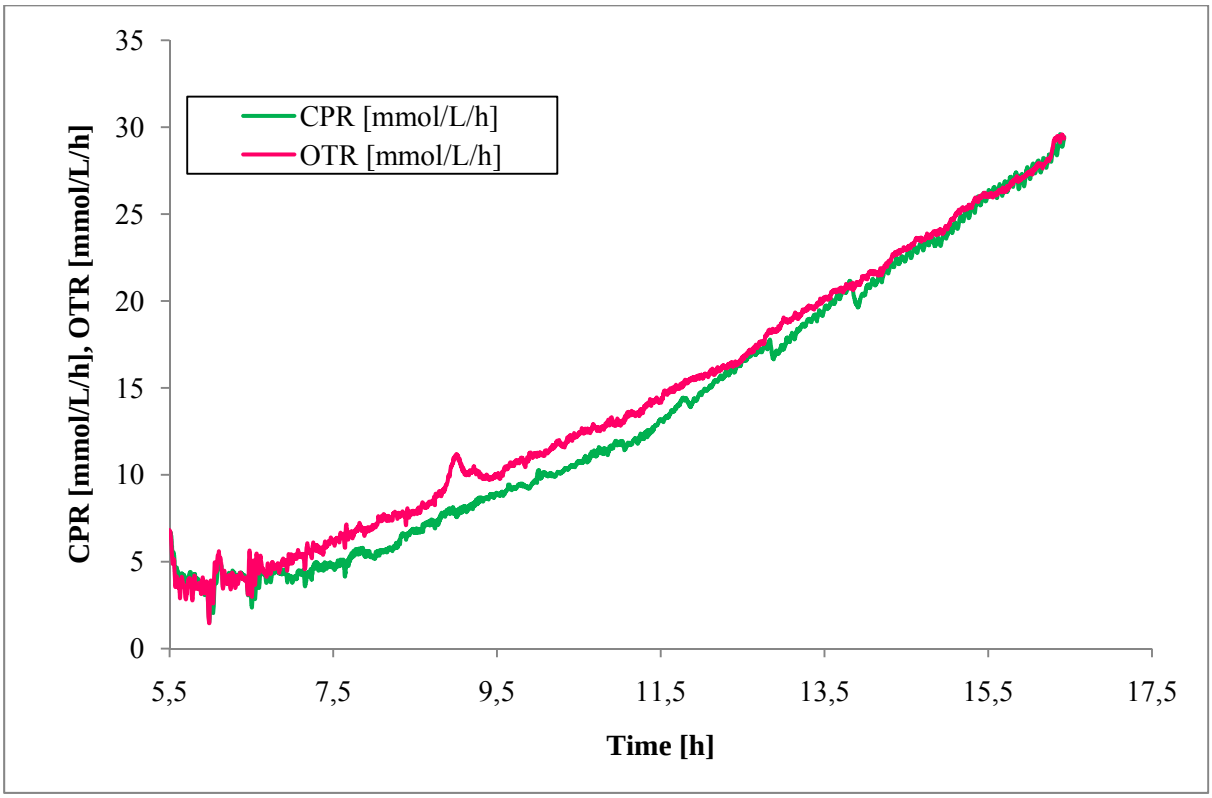

Figure 18: CPR and OTR during the cultivation of *E. coli* K1 at growth rate 0.15 h^{-1}.

Since the assigned growth rate is very less, and the feeding started after finishing up the whole amount of glucose available, the organism took more time to be get adjusted with the new environment and hence, too much disturbance in the beginning of the feeding phase. For convenience, the data from 5.5 h is shown in Figure 18.

The initial value of the biomass given to the software before starting the feeding procedure was 2.5 g/L and by the end of the feeding phase the estimated biomass value reached the

point of 12 g/L. The off-line value in the beginning was more or less the same as the value given to the software. The off-line value of the growth rate during this cultivation was 0.11 h^{-1}. It may be due to the higher yield coefficient given to the software that the difference between estimated and off-line biomass increases as the cultivation process proceeds. Figure 19 shows the off-line and on-line biomass during cultivation 3.

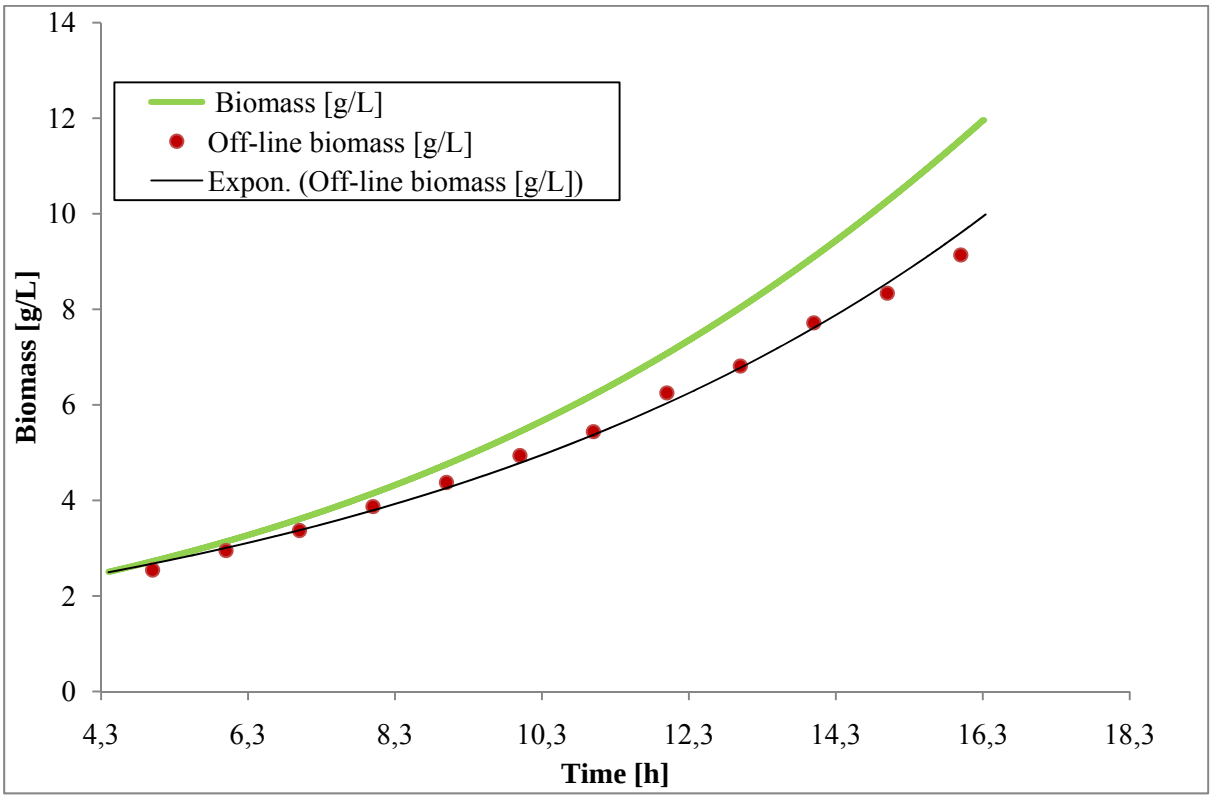

Figure 19: Off-line and on-line biomass during the cultivation of *E. coli* K1 with growth rate 0.15 h^{-1}.

Since the cultivation has been carried out under glucose limited condition, the probability of acetate formation is nullified. As expected glucose and acetate values were always zero throughout the cultivation. So it is not plotted in the figures. In glucose limited conditions, the bacteria would have consumed acetate in the absence of glucose which results in zero acetate concentration. In Figure 20, it is clear that at growth rate 0.15 h^{-1}, the PSA and biomass increases with the progress of the cultivation. The PSA amount reached a value of 1.48 g/L at the end of the feeding phase. Thus the cultivation provides the result that the biomasses yield was 0.33 g/g where as the PSA yield was 0.05 g/g when calculated using estimated values. The biomass and PSA concentration during the cultivation is plotted in Figure 20.

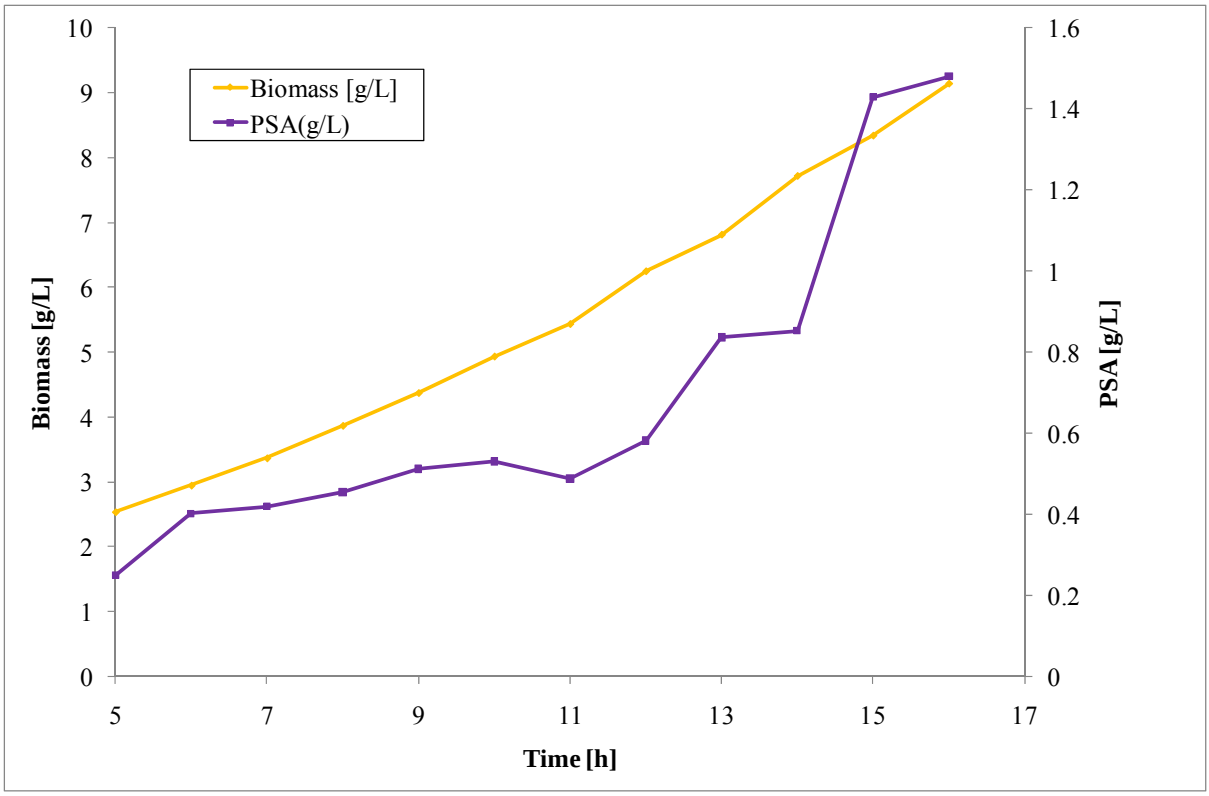

Figure 20: Biomass and PSA concentration during the cultivation of *E. coli* K1 with growth rate 0.15 h^{-1}.

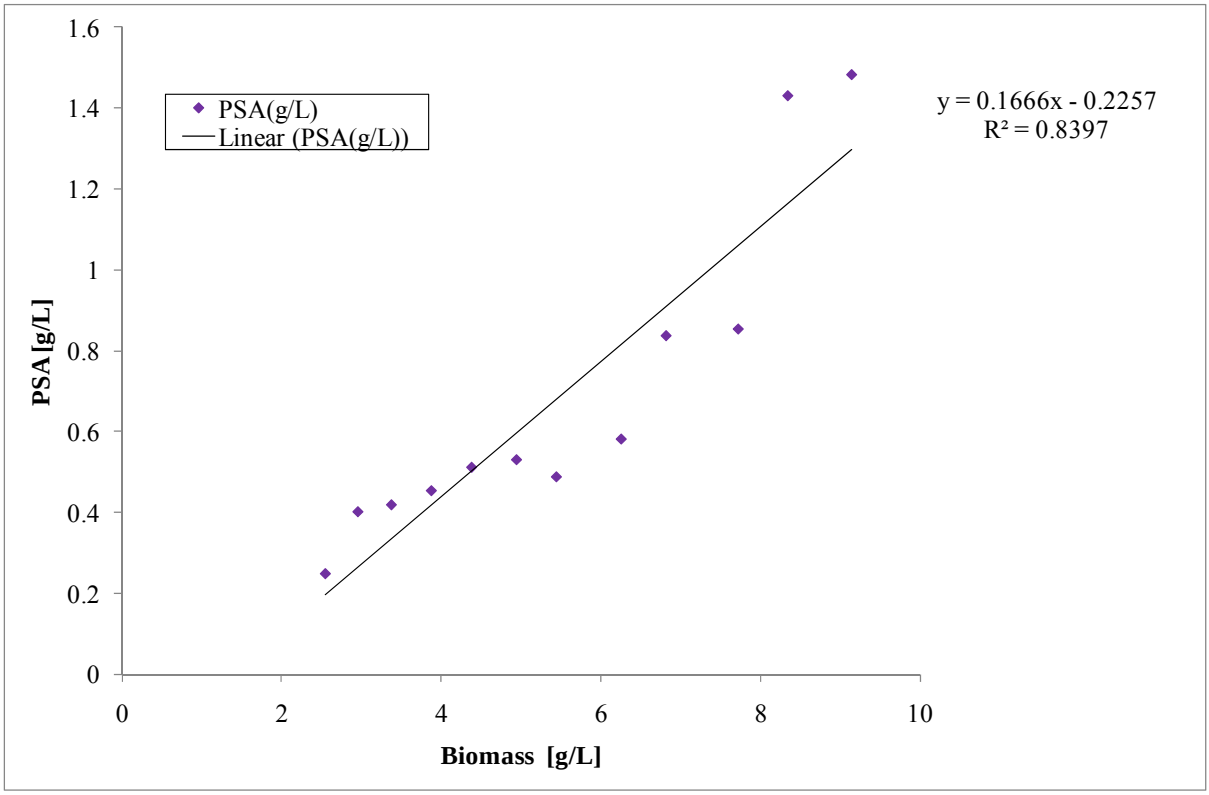

Figure 21: Plot of PSA against biomass during feeding phase of *E. coli* K1 cultivation at growth rate 0.15 h^{-1}.

A graph is plotted with PSA concentration during the feeding phase of the cultivation against biomass in Figure 21. The slope of the graph is 0.167 $g_{PSA}/g_{Biomass}$.

4.1.2.2. Cultivation 4

To study the effect of a higher growth rate on the production of biomass and polysialic acid, cultivation has been done at a growth rate of 0.25 h^{-1}.The procedure of the cultivation is the same as that followed in cultivation 3. Here, also the batch phase was started with an initial glucose concentration of 5.0 g/L. The feeding had started after the glucose concentration reached zero value. In this particular cultivation, the batch phase continued for 4.9 hours and the feeding phase continued for the next 9.3 hours. Till 11^{th} hour, the software was keeping the growth rate as 0.22 h^{-1} even though the given value to the software was 0.25 h^{-1}. After that point the real biomass shows a negative deviation and the growth rate sink to a value of 0.18 h^{-1}. The exhaust gas analysis has shown an uninterrupted growth throughout the feeding phase. Figure 22 shows the CPR as well as the OTR data during this cultivation.

The initial value of biomass given to the software was 2.6 g/L and finally at the end of the cultivation, the estimated value reached a value of 13.9 g/L. The off-line value of biomass in the beginning of the cultivation was 2.6 g/L which was the same as the initial value given to the software. The estimated and measure biomass values were in exact agreement with each other till 11^{th} hour of cultivation and after that it showed a small negative deviation from the estimated values which can be seen in Figure 23. The final value of off-line biomass was 11.73 g/L. Here, the deviation is not as much as in the previous cultivation. This may be because the yield value given to the software was 0.39 g/g which is more or less the same as the original value.

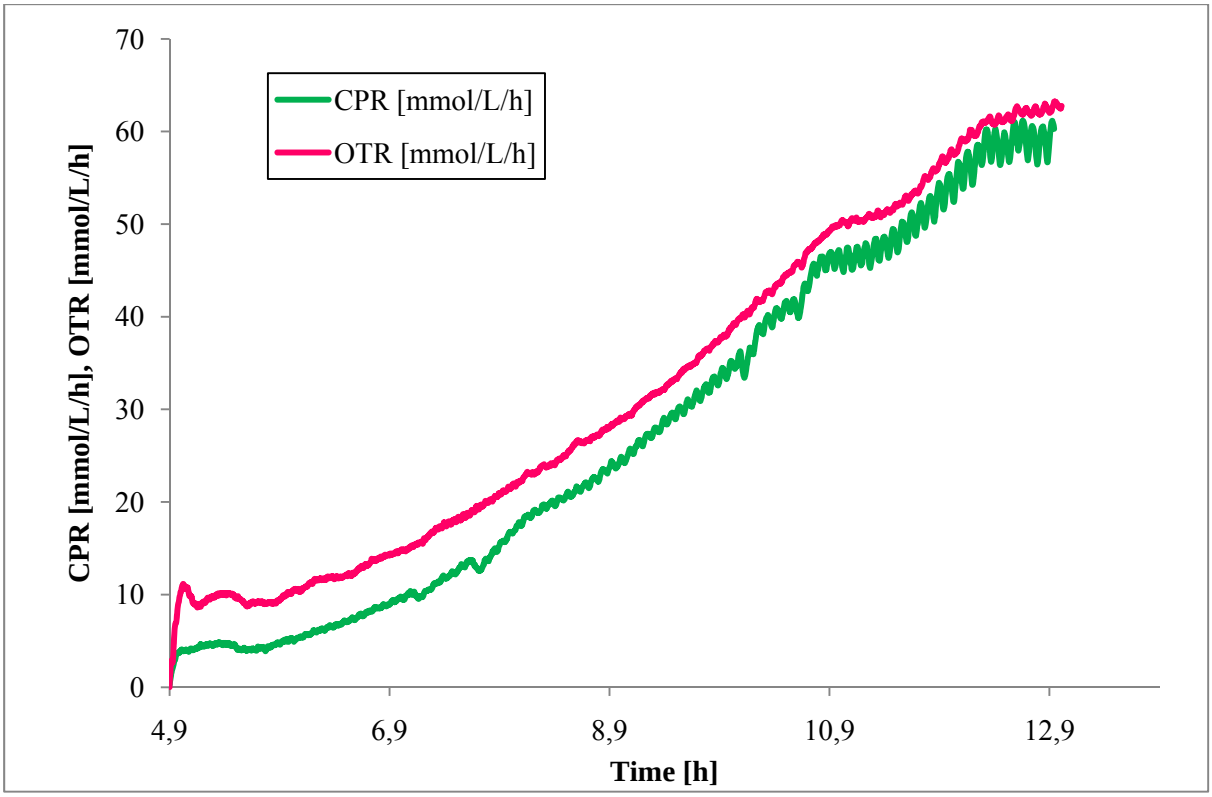

Figure 22: CPR and OTR during the cultivation of *E. coli* K1 at a growth rate of 0.25 h^{-1}

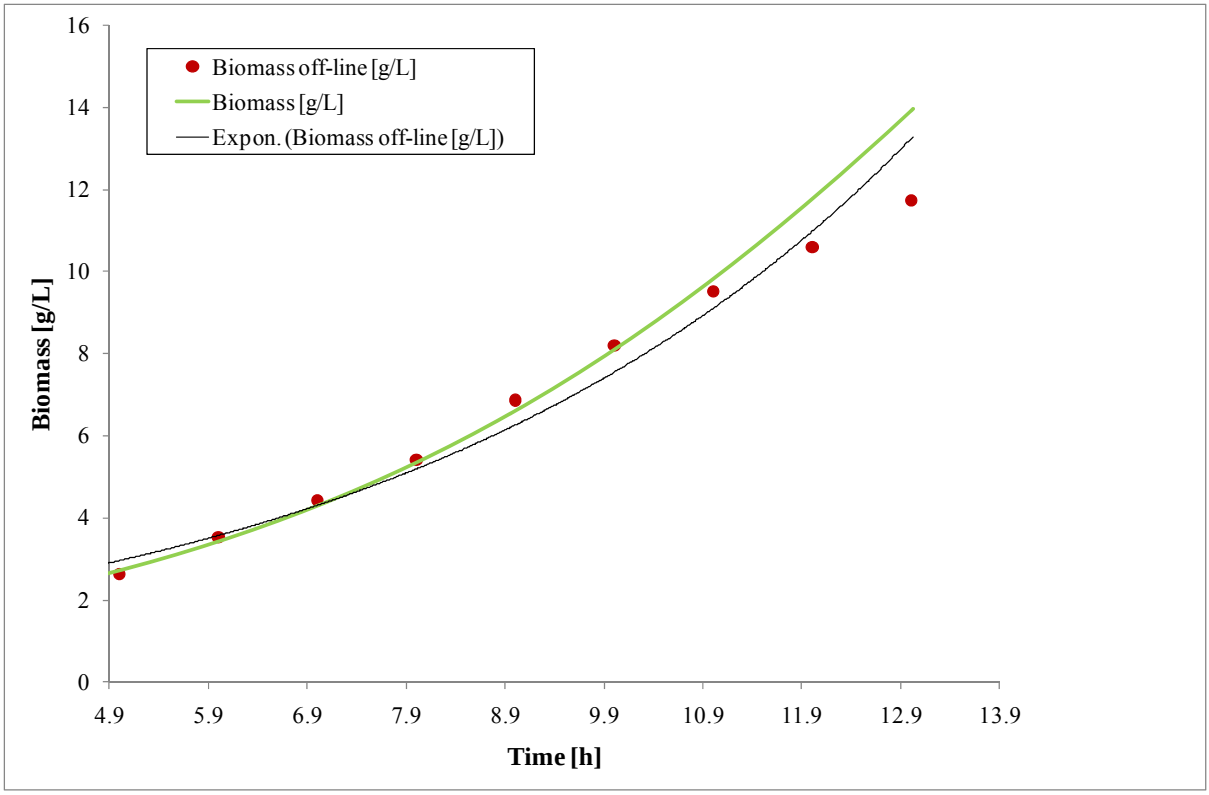

Figure 23: Estimated and measured biomass during the feeding phase of the cultivation of *E. coli* K1 with growth rate 0.25 h^{-1}.

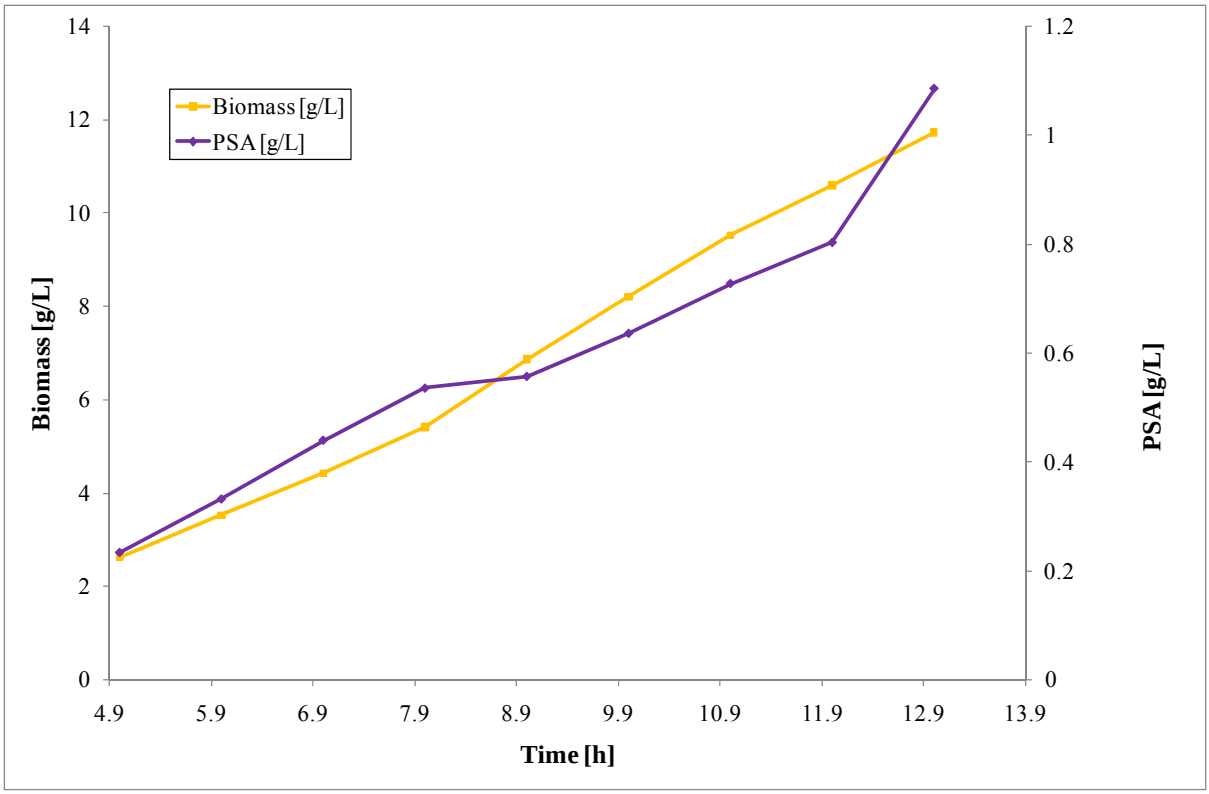

Figure 24: The biomass and PSA concentration during the cultivation of *E. coli* K1 with growth rate 0.25 h^{-1}.

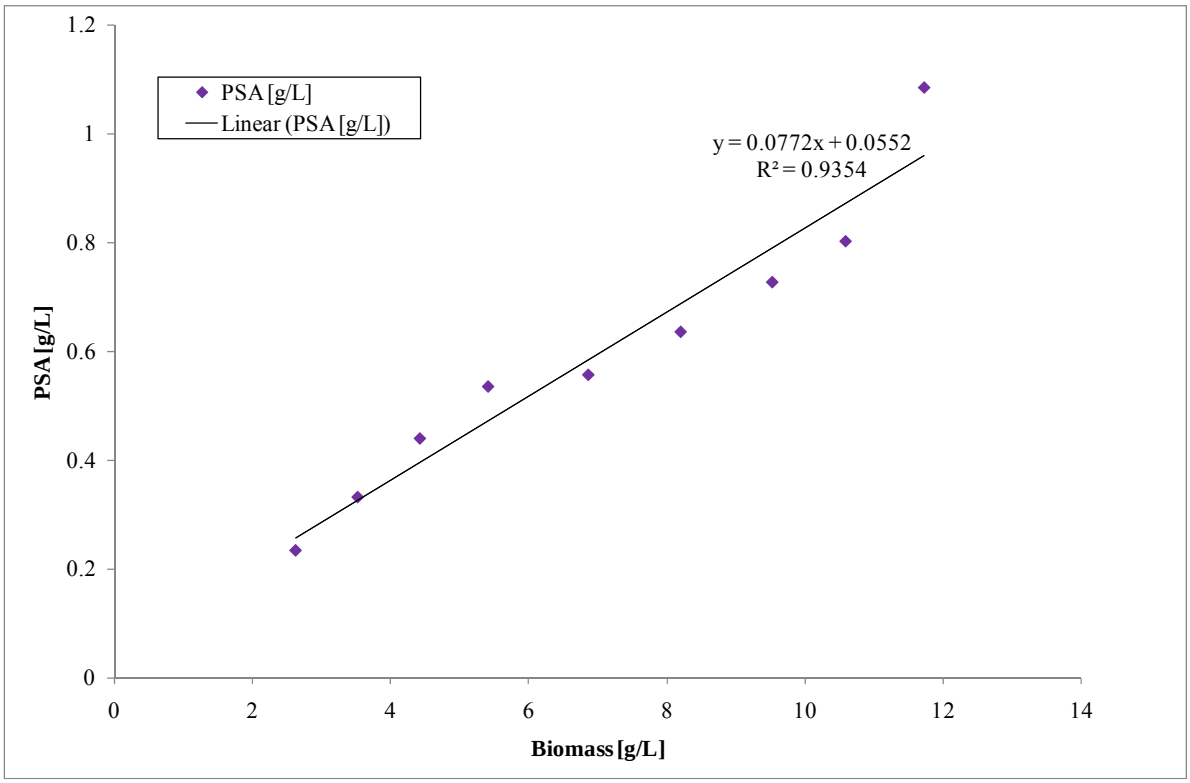

Figure 25: PSA concentration plotted against biomass concentration during the cultivation of *E. coli* K1 at growth rate 0.25 h^{-1}.

In Figure 24 the concentration of PSA as well as biomass during the feeding phase of the cultivation is presented. The biomass and PSA yield calculated using theoretical values were 0.4 g/g and the 0.031 g/g respectively in this cultivation which is found to be better than the value at growth rate 0.15 h^{-1}.

As presented in Figure 25, the slope of biomass-PSA curve is 0.08 $g_{PSA}/g_{Biomass}$ and the intercept is 0.06 g_{PSA}/L. Here, it is clear that PSA concentration is proportional to biomass concentration.

4.1.2.3. Cultivation 5

In this chapter, an aerobic glucose limited fed-batch cultivation of *E. coli* K1, at a growth rate of 0.35 h^{-1} is discussed in detail. This cultivation includes a pre-culture phase, a batch phase and a feeding phase. The pre-culture medium is LB medium and its composition is specified in appendix A6. The batch phase was started with an initial glucose concentration of 5 g/L. Off-line samples were taken to analyse for biomass, acetate, OD, glucose and PSA. The exhaust gas analysis also was performed throughout the cultivation.

In the same way as mentioned in the previous cultivations, the software was keeping the growth rate at a lower value in this cultivation also. In this cultivation the intended value of growth rate was 0.35 h^{-1}, but the calculated growth rate using off-line biomass measurements is 0.32 h^{-1}.

The feeding phase of the cultivation was started when the glucose concentration inside the bioreactor fell down to 0 g/L. Besides off-line glucose measurement, it was confirmed also by the exhaust gas analysis data. The CO_2 concentration showed a sharp decrease when the glucose concentration was zero inside. The feeding started with the help of exponential feeding software programme named as Neu-ORK programme.

The initial values of the parameters given to the software are summarised in Table 6 (Page 50). The cultivation was continued only till the system can maintain the dissolved oxygen concentration at 30 %. The CPR and OTR seem to be very good in agreement with each other. Data from 5 hours to 9h is shown in figure 26. CPR and OTR are increasing steadily till the end of the feeding phase.

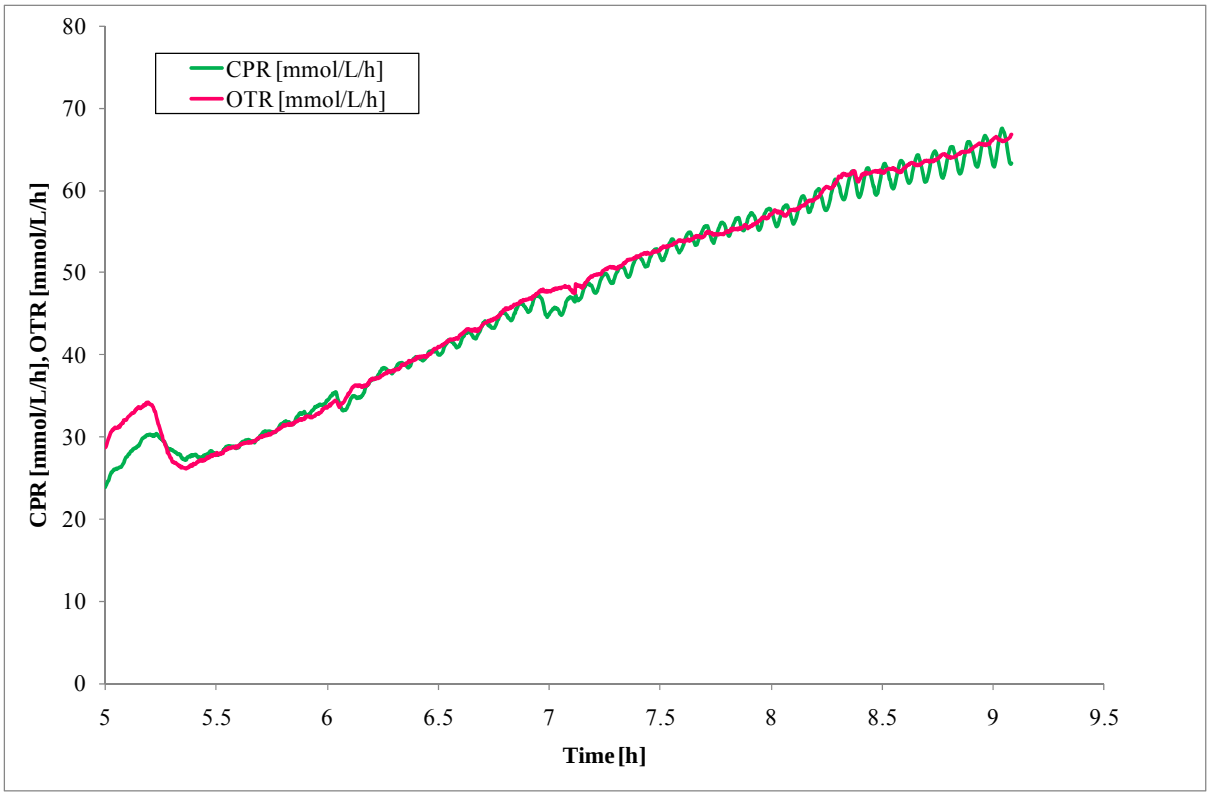

Figure 26: CPR and OTR during the feeding phase of the cultivation of *E. coli* K1 at growth rate 0.35 h^{-1}.

Even in glucose limited cultivation condition, the organism was growing uninterrupted till it reached the end of the cultivation. The feeding process continued for 4.4 hours. The initial value of biomass given to the software was 3.6 g/L. The estimated biomass value at the end of the cultivation reached a point of 13.42 g/L. But the off-line value was less than the estimated value. The off-line measurements gave a biomass concentration of 10.27 g/L only. This may be due to the difference in the initial value of biomass given to the software. The given value to the software was below the actual biomass value inside the bioreactor, i.e., about 2.9 g/L. When the real value of 0.32 h^{-1} is given to the growth rate, then, the biomass curve is more or less the same as off-line values (see Figure 27).

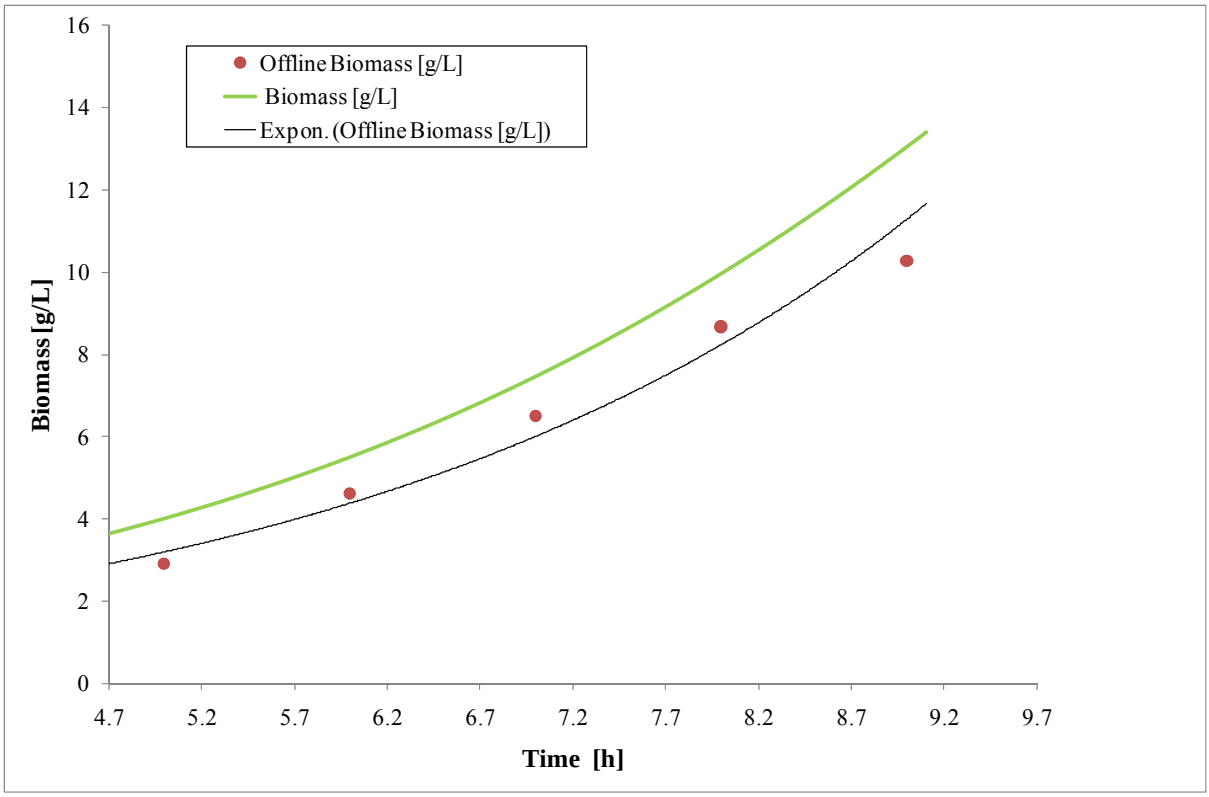

Figure 27: The estimated and measure biomass data during the cultivation of *E. coli* K1 at a growth rate of 0.35 h^{-1}.

The glucose and acetate data are not plotted in the Figure 27 because they were found to be at 0 g/L all the time. Since the bacteria were growing in glucose limited conditions, they would utilize the acetate if it was formed when they lack glucose source. However, towards the end of the feeding phase a PSA concentration of 1.12 g/L is obtained.

Thus at the end of this particular cultivation which was carried out under aerobic glucose limited conditions, the biomass yield was 0.36 g/g and the PSA yield was 0.043 g/g. Here, PSA yield is comparably higher than that in cultivation 3 and 4.

Figure 28 showing the biomass and PSA concentration during the feeding phase proves that PSA and biomass concentration increasing continuously till the end of the feeding phase. The dependence of PSA and biomass is presented in Figure 29. Compared to the other cultivations carried out with exponential feeding profile the slope of the linear regression line has the highest value.

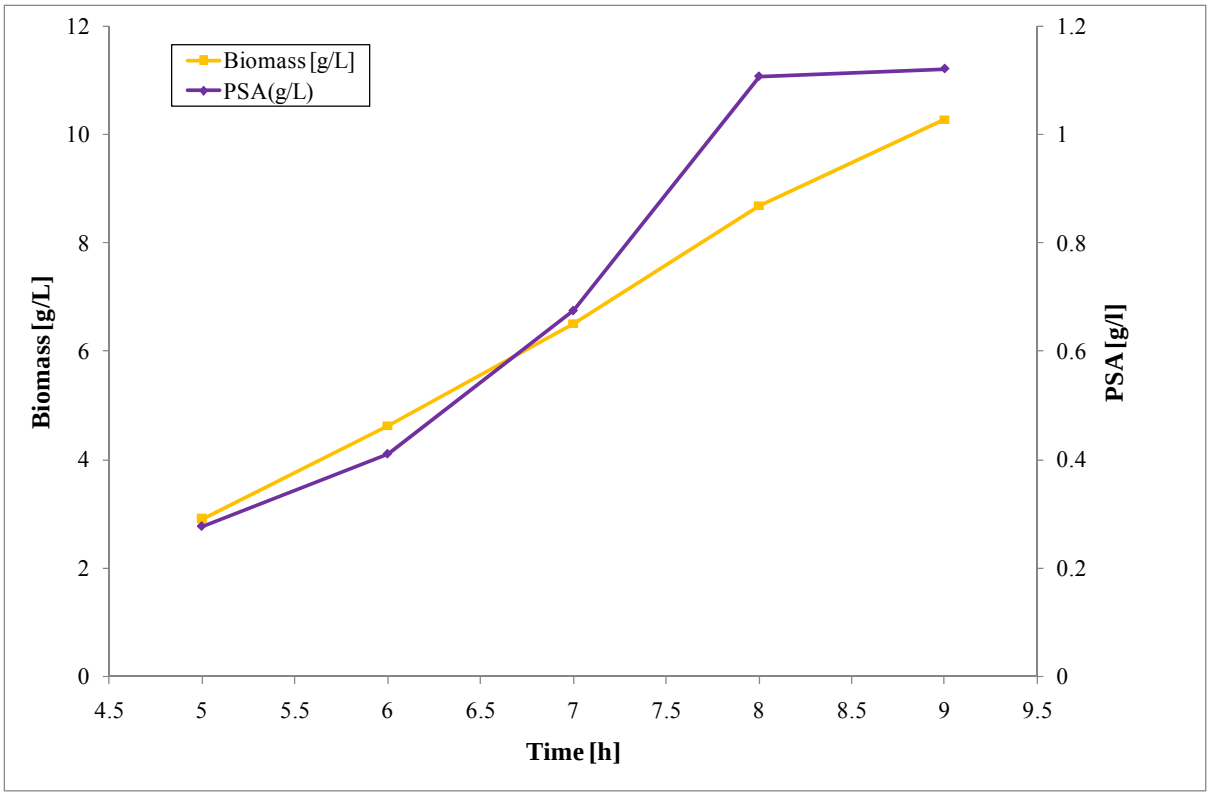

Figure 28: Biomass and PSA concentration during the cultivation of *E. coli* K1 with growth rate 0.35 h^{-1}.

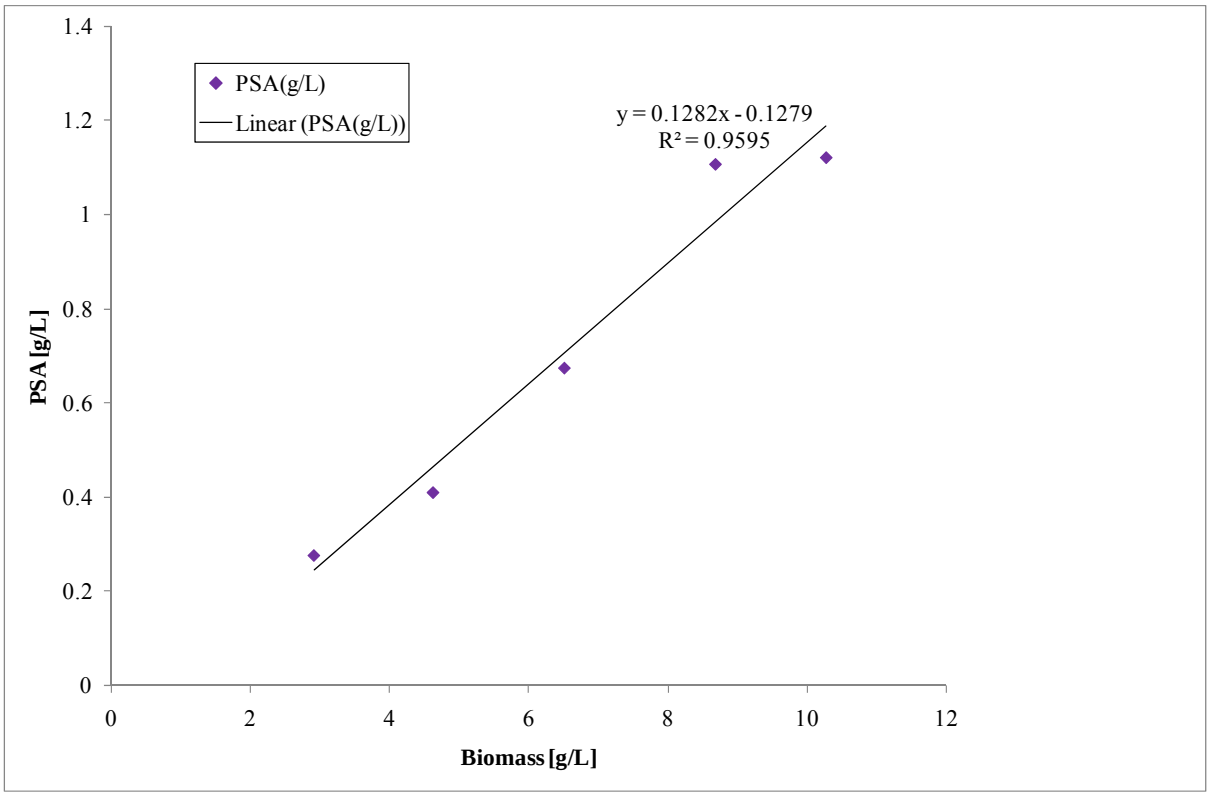

Figure 29: PSA against biomass during the cultivation of *E. coli* K1 at growth rate 0.35 h^{-1}.

4.1.2.4. Comparison of cultivations at different growth rates

The fed-batch cultivation of *E. coli* K1 has been carried out under different growth rates. The growth rates under consideration are 0.35 h^{-1}, 0.25 h^{-1} and 0.15 h^{-1}. Since the cultivations were carried out under glucose limited conditions, no acetate is produced during the feeding phase. The off-line analysis revealed that the glucose concentration was always zero inside the bioreactor. Thus the feeding mode was functioning well and the parameters got adjusted to the small disparities in the initial values. In cultivation 3 where the growth rate was maintained at 0.15 h^{-1}, the system took a longer time to be get adjusted with the new environment when the feeding started. An overview of the yield factors are given in Table 7.

Table 7: PSA yield and biomass yield during cultivations of *E. coli* K1 at three different growth rates with exponential feeding.

Parameter	**Cultivation 3**	**Cultivation 4**	**Cultivation 5**
Growth Rate [h^{-1}]	0.15	0.25	0.35
Biomass Yield [g/g]	0.33	0.4	0.36
PSA Yield [g/g]	0.05	0.031	0.043
PSA production rate [(g/L)/h]	0.1	0.1	0.26

The biomass yield didn't show any definite pattern of change according to the growth rate. The presented data shows that under glucose limited conditions, the difference in growth rate will not affect the biomass yield considerably. But the PSA yield is highest in the cultivation with lowest growth rate. When, considering PSA yield and production rate together, the production rate is more than double, when the growth rate changed from 0.15 h^{-1} to 0.35 h^{-1}. At the same time the yield factor is not so much different. Since the production rate is extremely lower in cultivation 3, it cannot be taken as a good condition for PSA production process.

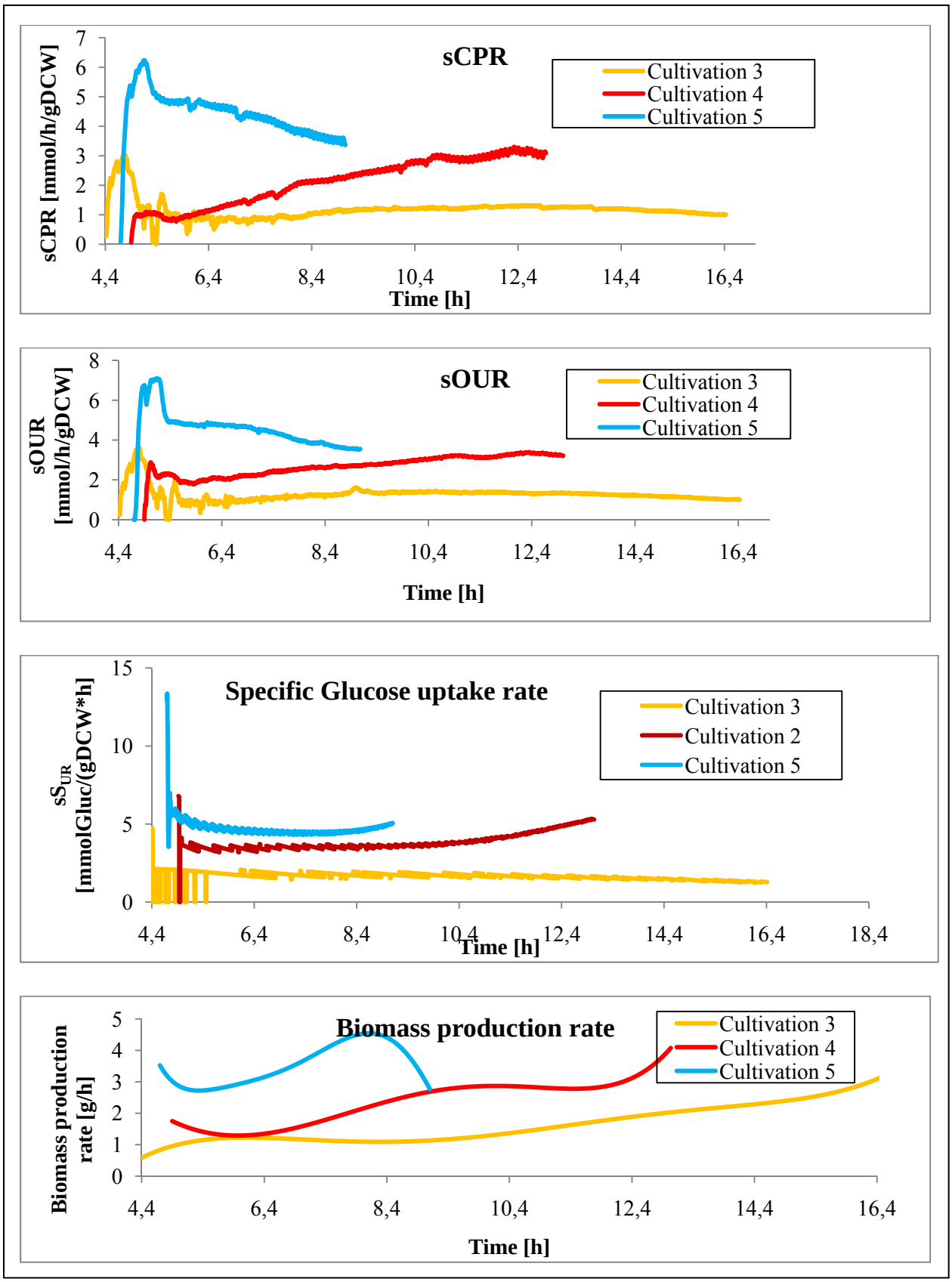

Figure 30: Comparison of specific CPR, specific OTR, specific glucose uptake rate and biomass production rate during feeding phase of cultivations using exponential feeding.

Figure 30 illustrates the comparative evaluation of specific CPR, specific OTR, specific glucose uptake rate and biomass production rate during exponential feeding cultivation at different growth rates. In cultivation 3, the bacterial cells are growing under very low growth rate of 0.15 h^{-1}, and the CPR and OTR show a steady pattern. This indicates a stable metabolic state of the *E. coli* cells. The biomass production rate is also increasing gradually. But in cultivation 4, one could observe a gradual increase in CPR, OTR, glucose uptake rate and biomass production rate. But all the changes are similar in pattern, which means that the cells are growing steadily. The off-line and estimated data are in good agreement. But the specific CPR and specific OTR shows continuous decreasing pattern while the glucose uptake rate is gradually increasing. This might be due to the inaccuracy of the initial biomass concentration given to the software. The given value was 3.6 g/L whereas the real off-line value was 2.9 g/L. But the feeding follows the given value of 3.6 g/L. Therefore, glucose availability per cell increases and the bacterial cells grow faster. More carbon flux flows to biomass build up. This could explain the CPR and OTR curve.

In order to study the carbon flux distribution, a carbon balance is made. From the elemental composition of biomass, an average value of 42.77 % is used as the carbon content of the biomass. The carbon balance data is given in Table 8.

Table 8: Carbon balance data of cultivations with exponential feeding

Cultivation	Carbon content of the biomass [%]	Carbon taken in [g]	Carbon coming out [g]	Ratio [C_{out}/C_{in}]
C3	42.77	14.12	11.36	0.81
C4		18.21	14.48	0.80
C5		11.02	8.76	0.80

The carbon recovery index is almost same in all the cultivations. Even then, the ratio is less than expected which indicates that the carbon recovery is not as much satisfactory in all these three cultivations.

Table 9: Carbon flux distribution in fed-batch cultivations of *E. coli* K1 with exponential feeding

Cultivation No	Biomass [%]	CO_2 [%]	Acetate [%]
C3	60.2	39.8	0
C4	65.5	34.5	0
C5	69.1	30.9	0

The carbon flux distribution presented in Table 9 shows that the carbon flow to biomass is increasing as the growth rate increases. The availability of more glucose per cell happened in cultivation 3 and the associated rapid growth can be proved by the fact that the percentage of carbon flowing to the biomass is higher in cultivation 3.

4.1.3. Fed-batch cultivations with EKF control

In the cultivations mentioned in this chapter, the glucose concentration was maintained at a constant desired level. The procedure for the cultivation process is the same as explained in chapter 3.2 (Page 28). Cultivation of *E. coli* at different glucose set points were performed using a control system based on flow injection analysis (FIA) complemented by an extended Kalman filter. Mainly two cultivations are discussed here with glucose set points of concentrations 0.05 g/L and 0.1 g/L. The objective for selecting these set points was to study the dependence of biomass production at different constant glucose concentrations. In all the cultivations, the controller has started towards the end of the batch phase, but before the batch comes to an end. The feeding phase was started immediately after the glucose concentration reached the desired set points. The starting values for EKF during different cultivations are summarised in Table 10.

The cultivation process is explained in detail in the following subtopics. In all the successful cultivations, the control seems to be performing satisfactorily.

Table 10: Initial parameters given to EKF at different glucose set points

Parameters	Values at different glucose set points	
	Cultivation 6	Cultivation 7
S_{SP}	0.05 g/L	0.1 g/L
t_0	4.85 h	3.8 h
$X(t_0)$	2.45 g/L	0.99 g/L
$S(t_0)$	0.58 g/L	0.28 g/L
S_0	100 g/L	100 g/L
$V(t_0)$	1.5 L	1.69 L
$\mu_{max}(t_0)$	0.32 h^{-1}	0.4 h^{-1}
K_m	0.01 g/L	0.01 g/L
$Y_{X/S}$	0.325 g/g	0.3 g/g
R	0.01 g^2/L^2	0.01 g^2/L^2

4.1.3.1. Cultivation 6

In this cultivation, the glucose set point concentration was controlled at 0.05 g/L. This smaller glucose set point was selected to avoid the accumulation of acetate during the fed-batch cultivation, which can hinder the bacterial growth and in turn the product formation. The cells were maintained under completely aerobic condition throughout the cultivation so that the metabolism remained purely oxidative. The initial values for the estimation error covariance matrix *P* and the spectral density matrix of process noise *Q* were assigned to the Kalman filter as specified in Table 2 (Page 34). An overview of the initial parameters given to Kalman filter is depicted in Table 10. The sample flow rate for on-line detection of glucose concentration by FIA system was set as 0.036 L/h throughout the cultivation.

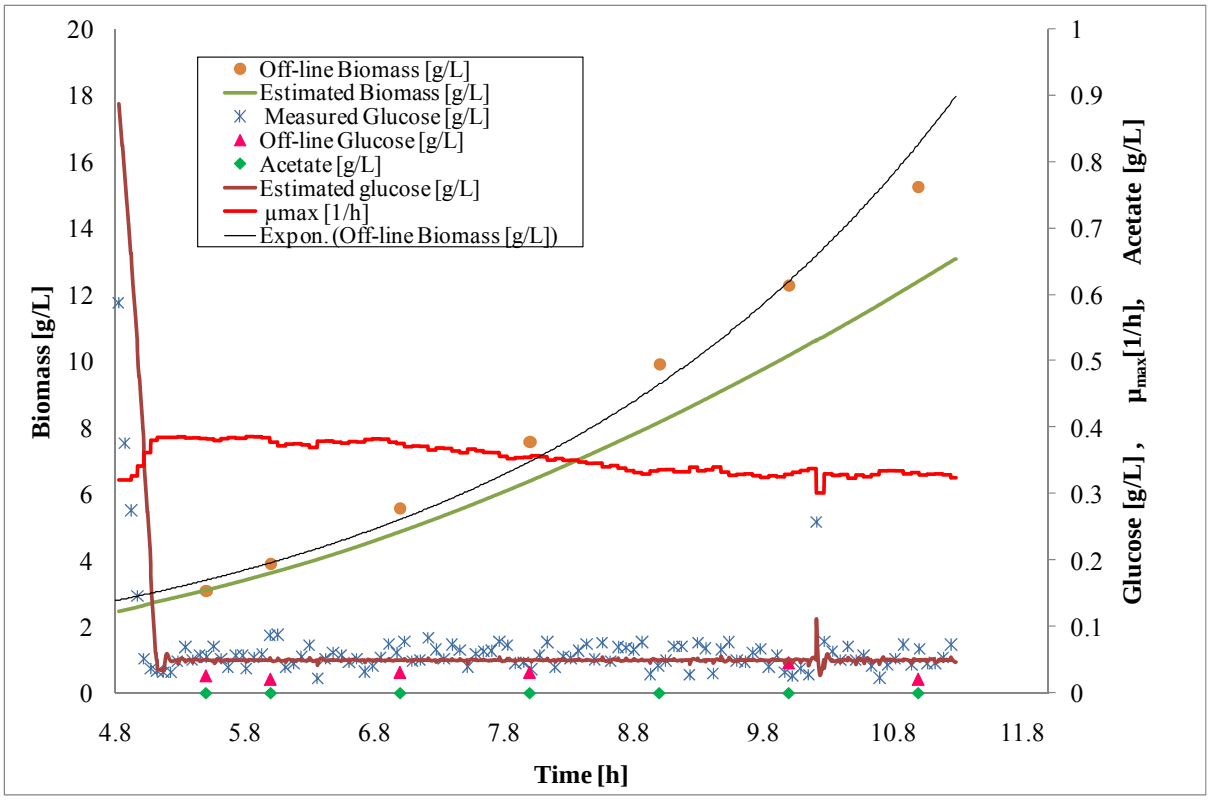

Figure 31: Estimated and measured glucose, biomass and maximal growth rate during the fed-batch cultivation of *E. coli* K1 at a set point of 0.05 g/L

The cultivation of *E. coli* at a glucose set point concentration of 0.05 g/L was started with batch phase having initial glucose concentration of 5 g/L. Kalman filter was started at 4.85 h when the glucose concentration inside the bioreactor was 0.58 g/L. Figure 31 demonstrates the estimated and measured biomass, estimated and measured glucose as well as maximal growth rate during the control phase of the cultivation process. The controller was able to maintain the glucose concentration around the set point throughout the feeding phase. The mean value of the measured glucose concentration was 0.054 g/L with a standard deviation of 0.015 g/L. This means that the standard deviation is smaller compared to the measurement error assigned in the Kalman filter. The maximum difference between the set point and the measured glucose concentration is 0.028 g/L. Followed by the addition of antifoam at 10.2 h, an abnormal value of measured glucose concentration is observed which is excluded from the data set while calculating the standard deviation. The estimated and measured concentrations correspond quite well. Another important point to be discussed here is the difference in on-line and off-line measured glucose concentrations. In all the measurements, the off-line glucose concentration lies below the set point. This can be attributed to the glucose consumption during the time delay in the off-line sample preparation and measurement even

though the samples were immediately transferred to the ice bath since the samples contain bacterial cells.

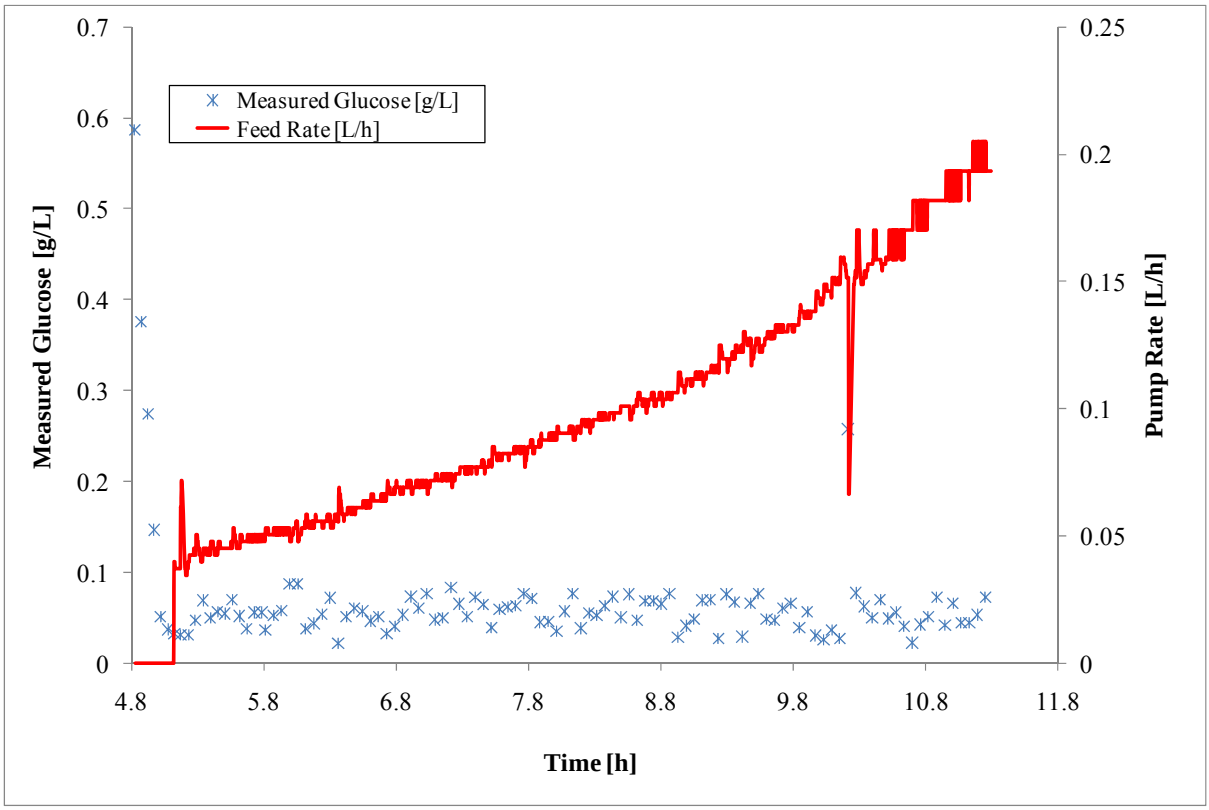

Figure 32: Measured glucose concentration and feed rate during the cultivation of *E. coli* K1 at glucose set point 0.05 g/L.

As stated above, an abnormal glucose concentration measurement is obtained at 10.2 h which amounted to a value of 0.25 g/L due to the addition of antifoam. It is evident that the pump rate decreased instantly to bring down the glucose concentration to the set point (Figure 32). This proves that the controller can identify the critical situation very fast and can adjust accordingly to sustain the desired conditions.

On comparison of the estimated and measured biomass concentration, one could see that as the cultivation proceeds, the direction of the measured values exhibit an upward deviation from the estimated values. This can be due to the inaccurate yield factor value allocated to Kalman filter. The actual value of yield factor is 0.53 g/g whereas the given value to the Kalman filter was 0.325 g/g. In the final stages of the cultivation, the deviation reached a value of 2.84 g/L.

The pump rate of the feed solution is illustrated in Figure 32. One of the main advantages of this particular cultivation is that the pump rate never goes to zero even in the early stages of feeding phase. This is obtained by using tubing of very small internal diameter viz. 0.01 mm.

The initial value assigned for maximal growth rate was 0.32 h^{-1} which is less than the original value. EKF suddenly increases the estimated value of the growth rate to 0.37 h^{-1} which is kept more or less constant in the remaining part of the cultivation. The average value of the maximal growth rate estimated by Kalman filter is calculated to be 0.37 h^{-1} which is calculated after excluding the initial rising up part of the growth rate till 5.1 h of the cultivation. This indicates that the bacteria is getting adjusted to the surroundings so fast when the set point is lower.

The CPR and OTR values during the fed-batch cultivation are illustrated in Figure 33. This shows that the cultivation encounters no limitation of any nutrient or oxygen for maintaining the growth. The large fluctuation at 10.2 h is due to the addition of antifoam.

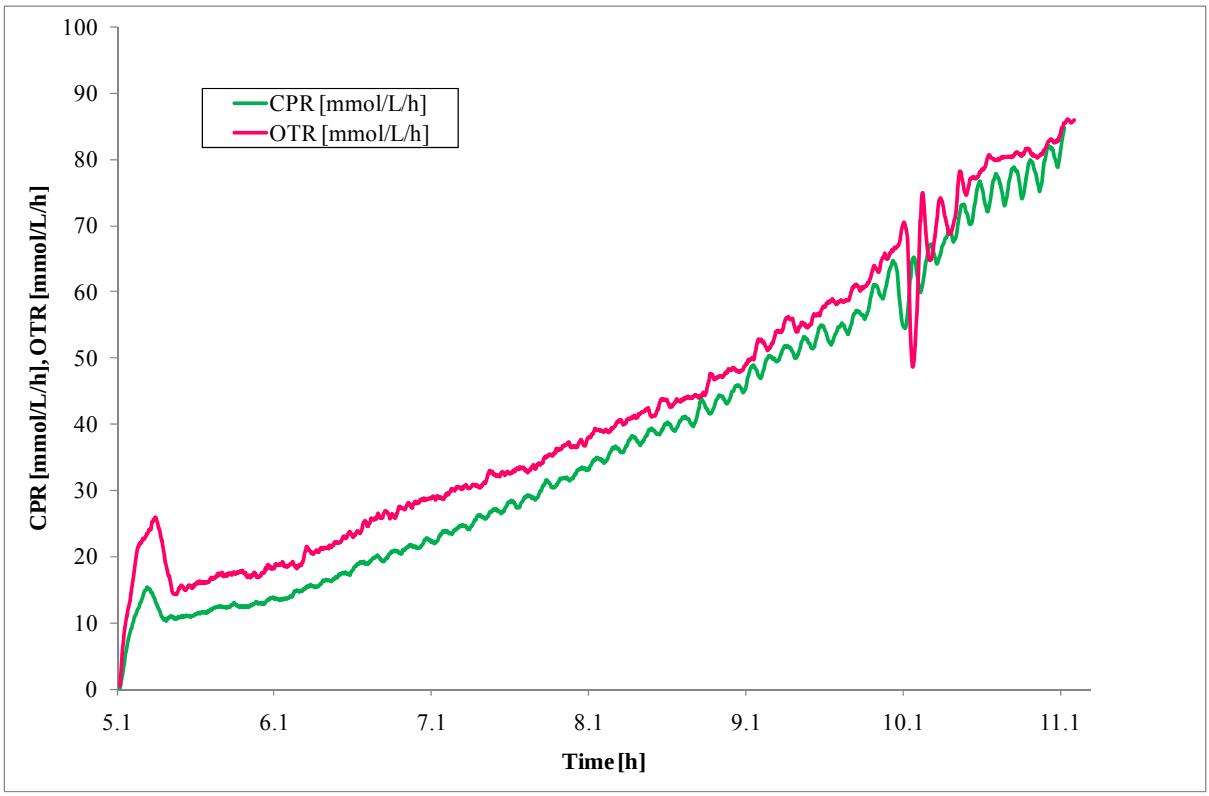

Figure 33: CPR and OTR in the feeding phase of *E. coli* cultivation at glucose set point 0.05 g/L; at 10.2 h antifoam was added which made fluctuation in CPR and OTR.

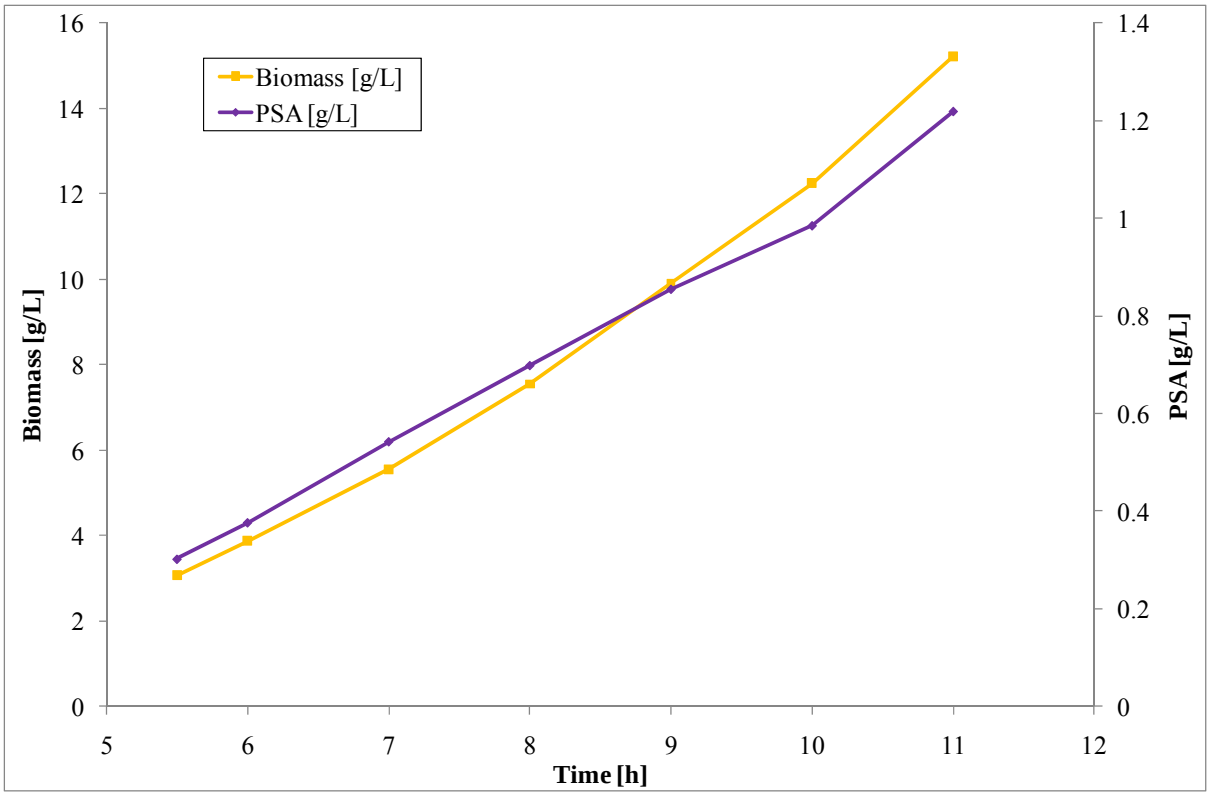

Figure 34: Biomass and PSA concentration during the cultivation of *E. coli* K1 at glucose set point 0.05 g/L.

Detectable amount of acetate is observed only in the beginning of the cultivation. This comes from pre-culture which was carried out in shaker flask. The possibility of oxygen limitation is high under this condition and so acetate formation occurs. But in the feeding phase, the glucose concentration is maintained at a lower level and the entire cultivation is under aerobic condition. So there is no possibility for either oxygen limitation or overflow metabolism. The cells are purely under aerobic conditions.

Another aim of reducing the glucose set point concentration was to study the effect of set point on the yield of the product i.e., polysialic acid. It is evident that the yield factor is maximized when the set point is lower. With this cultivation of glucose set point 0.05 g/L, a biomass yield of 0.53 g/g is obtained which is higher than the batch cultivations. PSA yield in this cultivation is 0.061 g/g. The biomass formation and product formation are depicted in Figure 34. It should be noted that PSA concentration is proportional to biomass concentration.

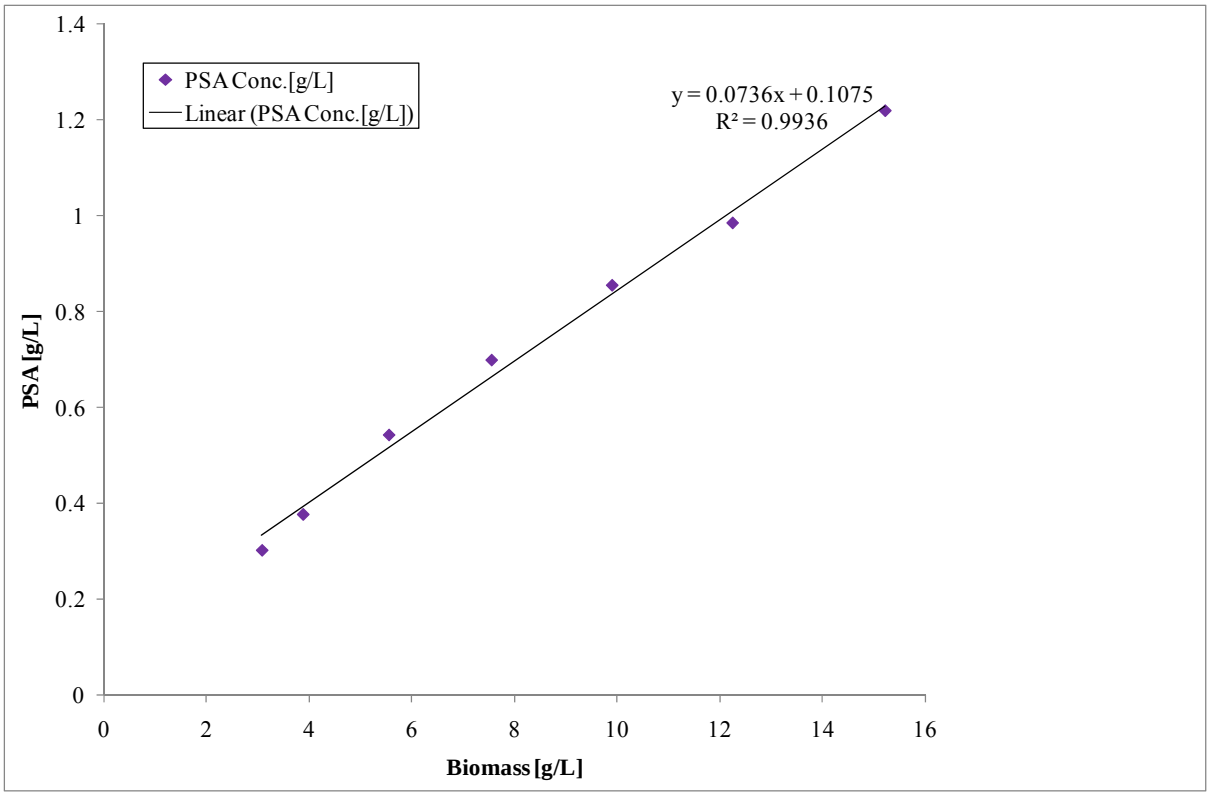

Figure 35: PSA against biomass during the cultivation of *E. coli* K1 at glucose set point 0.05g/L.

In Figure 35 one can see the plot of PSA concentration against biomass. The linear relation among PSA and biomass concentration has a determination coefficient of 0.99.

Thus, it is proved that it is possible to control the glucose concentration at a desired lower level without acetate accumulation using this control system.

4.1.3.2. Cultivation 7

In this particular cultivation, a desired glucose set point value of 0.1 g/L was given for the feeding phase. The cultivation started with a batch phase having initial glucose concentration 3 g/L. The monitoring over the cultivation by the control system started towards the end of the batch phase at 3.8 h when the glucose concentration inside the bioreactor was 0.28 g/L. No starvation period preceded the control phase. The initial biomass concentration for the control phase was 0.99 g/L. The initial value of the EKF as well as the kinetic parameters are summarised in Table 10 (Page 65).

The initial values for the estimation error covariance matrix P and spectral density matrix of process noise Q are assigned as specified in Table 2 (Page 34). After the commencement of

the control system at t_0=3.8 h, the glucose concentration steeply came down in 0.16 h to the desired set point value of 0.1 g/L. The control system switched on the feed pump immediately when the glucose concentration just reached the set point concentration, so that the glucose concentration does not drop considerably under the adjusted set point. The composition of the feed solution used is given in appendix A.1. Figure 36 demonstrates the course of the measured and estimated glucose concentrations as well as the biomass. This figure (Figure 36) depicts the data of the control phase for 4 h starting from 3.8 h of the whole cultivation. The off-line measured glucose concentration lies altogether somewhat below the on-line measured and predicted glucose concentration. This is probably because of the fact that a part of glucose was consumed by the microorganisms that were remaining in the sample during sample processing. However, it is much more important to note that the off-line value of glucose concentration never lie above the on-line measured glucose concentration. If it happened so, it would be an indication for a non optimal functioning of the measurement and control system.

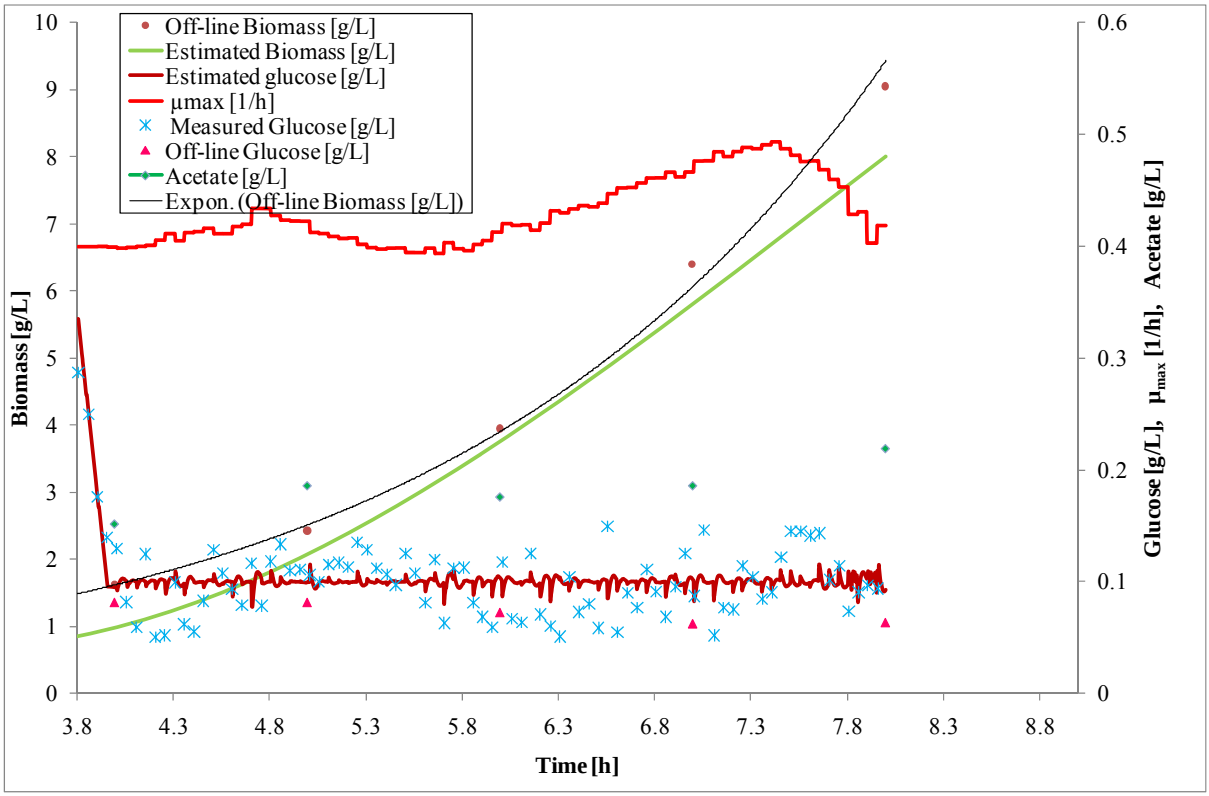

Figure 36: EKF data during the cultivation of *E.coli* K1 at a glucose set point of 0.1 g/L

The controller was able to adjust and to keep the glucose concentration at the set point of 0.1 g/L successfully. The average value of the measured glucose concentration of the feeding

phase was 0.097 g/L which was in good agreement with the desired set point concentration. The standard deviation was 0.027 g/L. This value is smaller compared to the assigned measurement error covariance *R* of the EKF. The off-line values of the measured biomass concentration lie above the predicted value till 8 h of cultivation. The initial higher values of the real biomass may be due to the slightly lesser initial value of biomass given to EKF.

During the feeding phase, the stirrer was set in cascade mode with stirrer speed varying in the range of 30 to 1200 rpm. The dissolved oxygen concentration was kept at 30 % throughout the feeding phase.

As could be observed in Figure 36, on the onset of feeding phase, the EKF adjusted the difference in maximum growth rate by increasing the value of 0.4 h^{-1} given to the Kalman filter to approximately 0.43 h^{-1}. The estimated maximal growth rate remains more or less constant till 6.2 h of cultivation and after that it started to increase slightly for about 1.2 hours and then came back to the original value. One can clearly observe that the estimated value of the maximal specific growth rate is always varying around the average value of 0.43 h^{-1}. The calculated value of the growth rate for the entire cultivation using the off-line biomass measurements is 0.46 h^{-1}. This may also cause the higher off-line biomass.

Figure 37 shows the curves for the CPR and OTR during the cultivation. The exponential pattern of the CPR and OTR evolution put forward a growth without any kind of limitation throughout the feeding phase.

Figure 38 shows the pump rate and the measured glucose concentrations. The pump rate was increasing exponentially as expected. One of the main advantage of this system is that, the on-line sample take out was carried out through a sampling module, which provides cell free sample stream and the measured glucose concentration is more or less the same as that inside the bioreactor. The flow rate of the on-line sample stream was fixed at 0.036 L/h. In this cultivation, the time delay in between sampling and assignment of new measured value to the Kalman filter was taken as 0.048 h. The Kalman filter would adjust the estimated value accordingly.

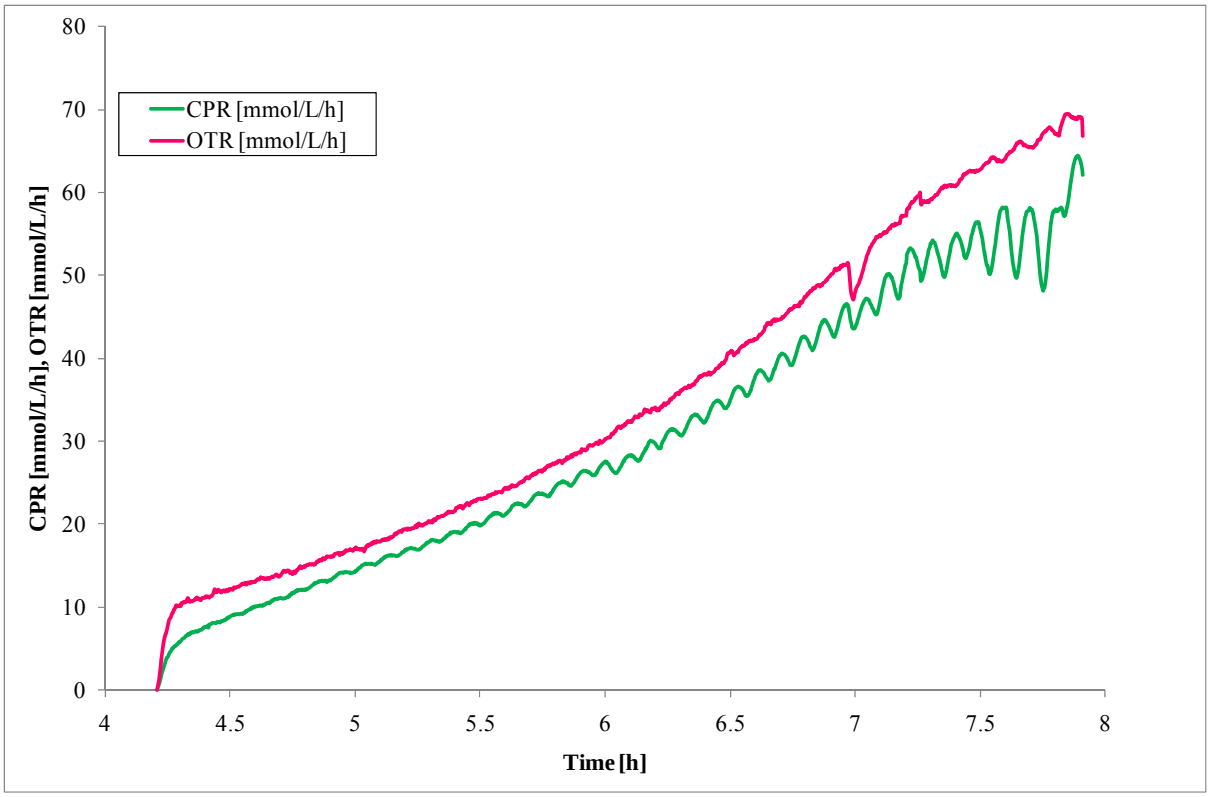

Figure 37: CPR and OTR during the cultivation of *E.coli* K1 at glucose set point 0.1 g/L

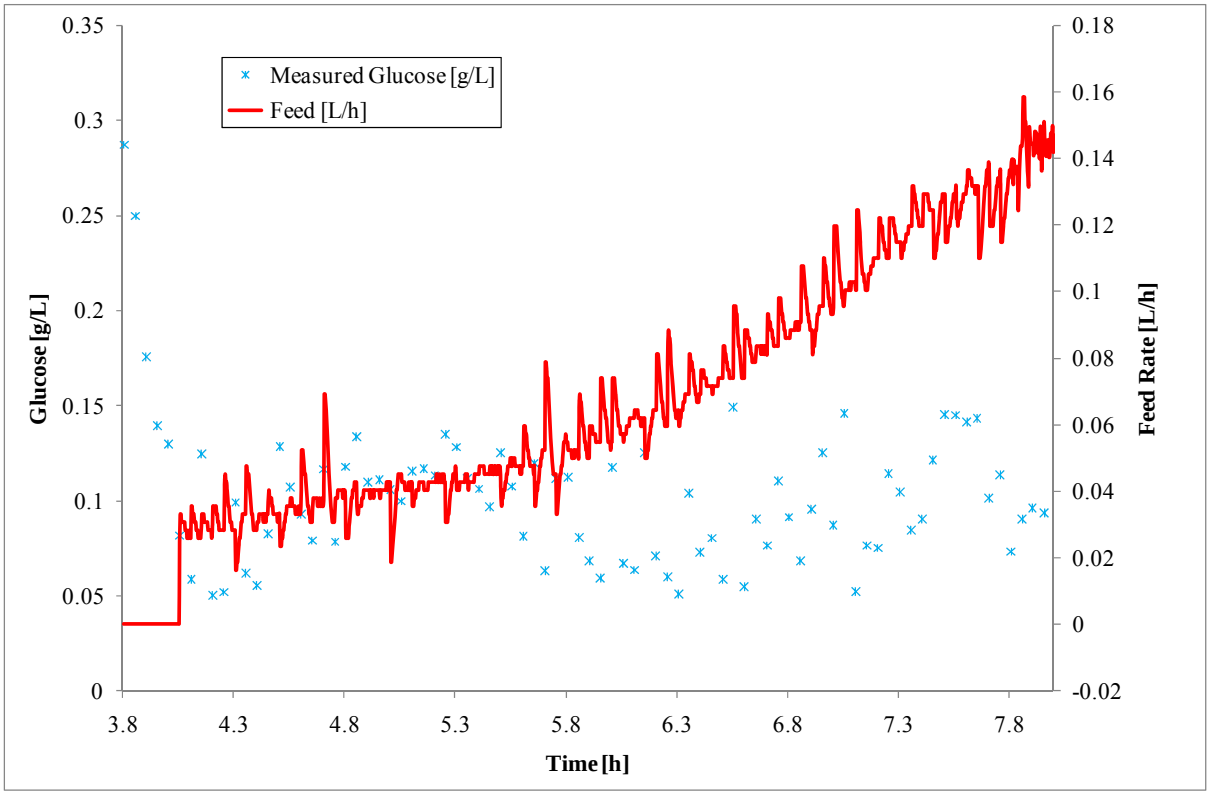

Figure 38: Pump rate and measured glucose concentration in fed batch cultivation of *E. coli* at set point 0.1 g/L.

At the end of the cultivation the biomass concentration reached a value of 9.07 g/L. It ultimately gives a biomass yield of 0.47 g/g. PSA yield is found to be 0.046 g/g. Figure 39 depicts the generation of biomass as well as polysialic acid in the feeding phase of the cultivation which is important in process efficiency point of view.

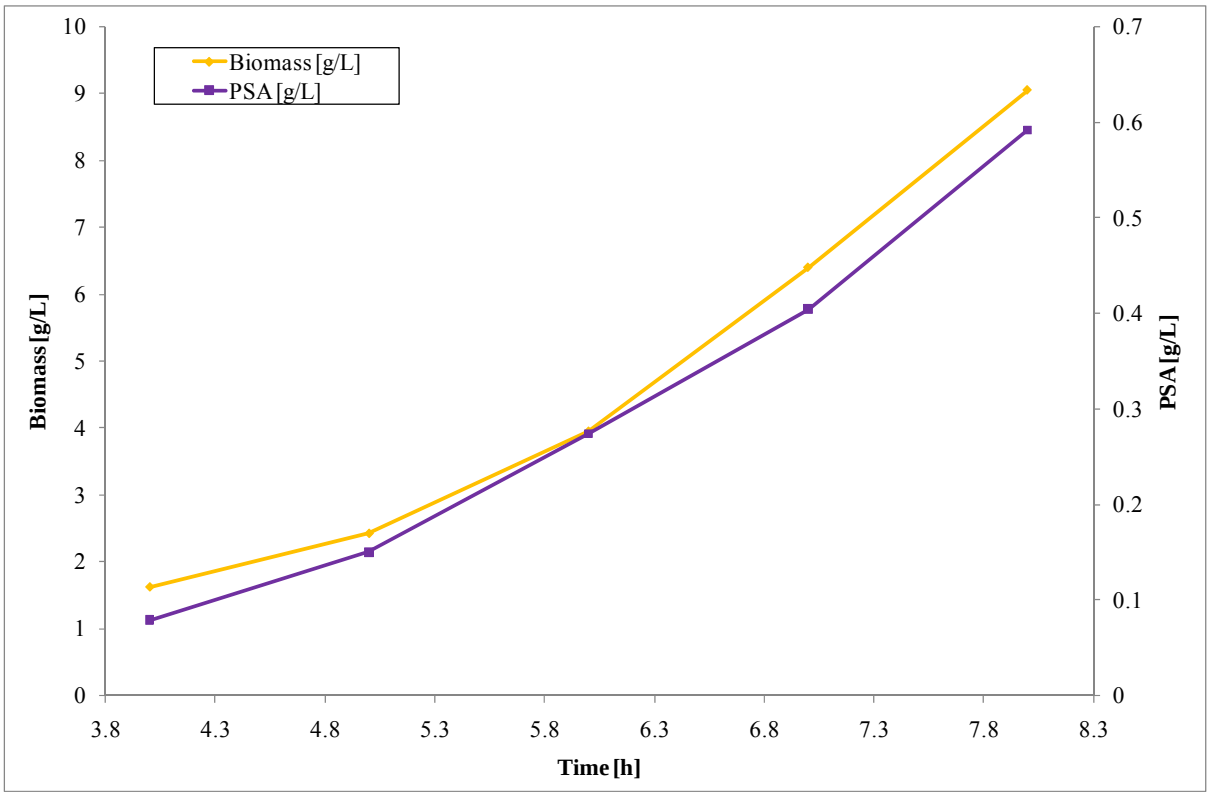

Figure 39: Production pattern of biomass and PSA in fed-batch cultivation of *E. coli* at set point 0.1 g/L.

Thus, the process gives a PSA yield of 0.046 g/g. Figure 36 (Page 71) gives an insight into the concentration of acetate during the process. Acetate from the batch phase exists in the feeding phase and at the end of the cultivation it reached a value of 0. 26 g/L which is not too high, but even then it is not negligible. Thus the evaluation of the entire process shows that 0.1 g/L glucose set point is not as good as the lower glucose set point of 0.05 g/L for the production of both biomass and polysialic acid. Figure 40 gives the relationship between biomass concentration and PSA concentration during the feeding phase of this cultivation.

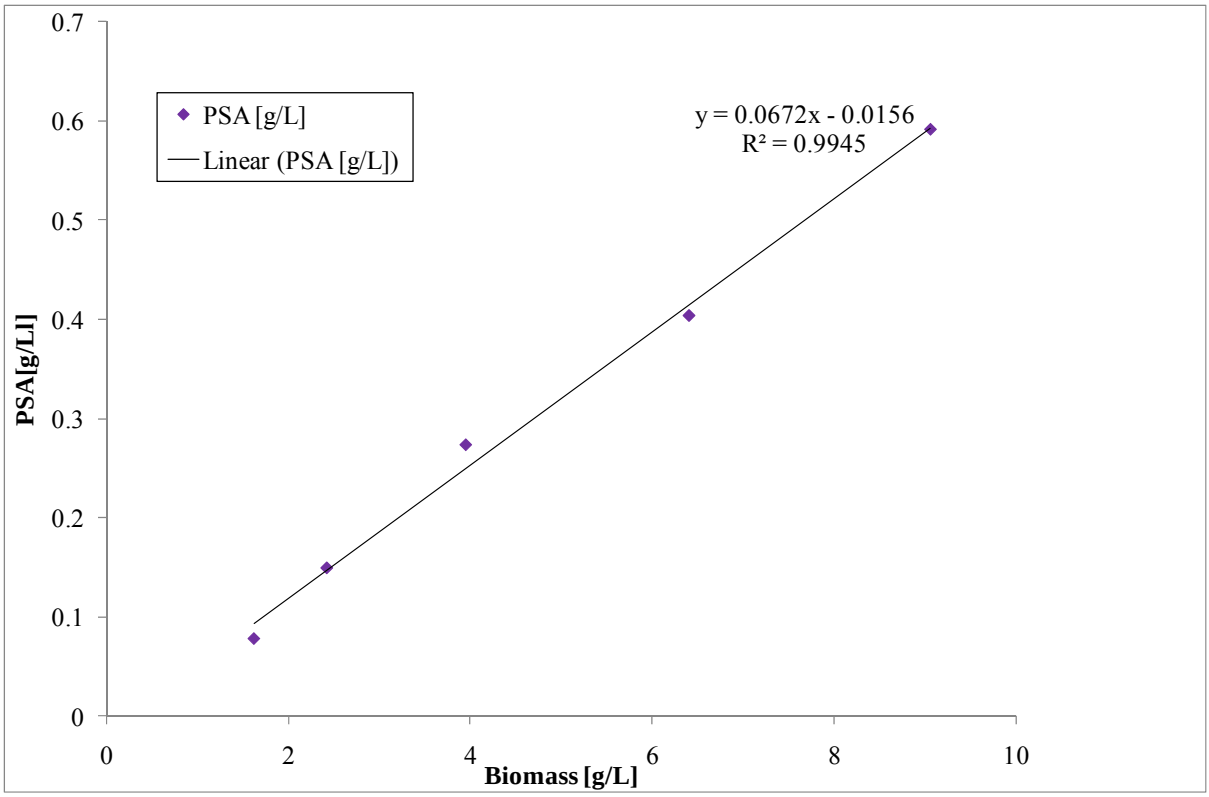

Figure 40: PSA against biomass during the cultivation of *E. coli* K1 at glucose set point 0.1g/L.

Here, again a linear relationship with a coefficient of determination of 0.99 is obtained.

4.1.3.3. Comparison of cultivations at different glucose set points

Two cultivations were performed at different glucose set points, namely, 0.05 g/L and 0.1 g/L. The controller was working good and was able to keep the desired glucose set point more or less stable. The standard deviation was also agreeable. The main results of the cultivations are presented in the Table 11.

It is evident that the maximum amount of biomass and PSA yield is obtained at glucose set point concentration of 0.05 g/L where no detectable acetate is found. The growth rate corresponding to this glucose set point is 0.37 h^{-1}. When the glucose concentration is controlled at 0.1 g/L, the estimated growth rate was higher than that at 0.05 g/L i.e., 0.43 h^{-1}. Then the bacterium produces detectable amount of acetate. Thus it is concluded that production of polySia get maximized when the glucose set point is minimum, but higher than zero.

Table 11: Comparison of cultivations of *E. coli* K1 at different glucose set points

Cultivation	**Cultivation 6**	**Cultivation 7**
Set Point	0.05 g/L	0.1 g/L
Growth rate	0.37 h^{-1}	0.43 h^{-1}
Biomass Yield	0.53 g/g	0.47 g/g
PSA yield	0.061 g/g	0.048 g/g
Acetate (Maximum amount obtained)	0 g /L	0.26 g/L

No starvation period occurred in these cultivations and therefore, the initial adjustment to the new feeding environment was not so complicated. Since a feed forward/feedback controller is used in the cultivation, the changes happening inside the bioreactor was instantly realised and the necessary steps were taken to keep the control at a stable state.

The biomass yield and the PSA yield shows that, as biomass increases, the PSA concentration also increases. But as the glucose set point increases, the bacteria produces more and more acetate so that a part of the carbon flux goes in that direction which results in a decrease of biomass yield. In Table 11 it is clear that the biomass yield was lower when the glucose set point was 0.1 g/L.

In order to have a better insight into the process, a comparative evaluation of specific CPR, specific OTR, specific glucose uptake rate and biomass production rate is shown in Figure 41. In cultivation 6 with glucose set point 0.05 g/L, specific CPR, specific OTR and specific glucose uptake rate remains stable till the end of the cultivation. It indicates that the bacterial cells maintain a stable metabolic state throughout the cultivation with gradual increase in the biomass production rate. The up taken glucose is fruitfully converted to biomass rather than to carbon dioxide in this cultivation. This is further confirmed by the absence of acetate during the cultivation.

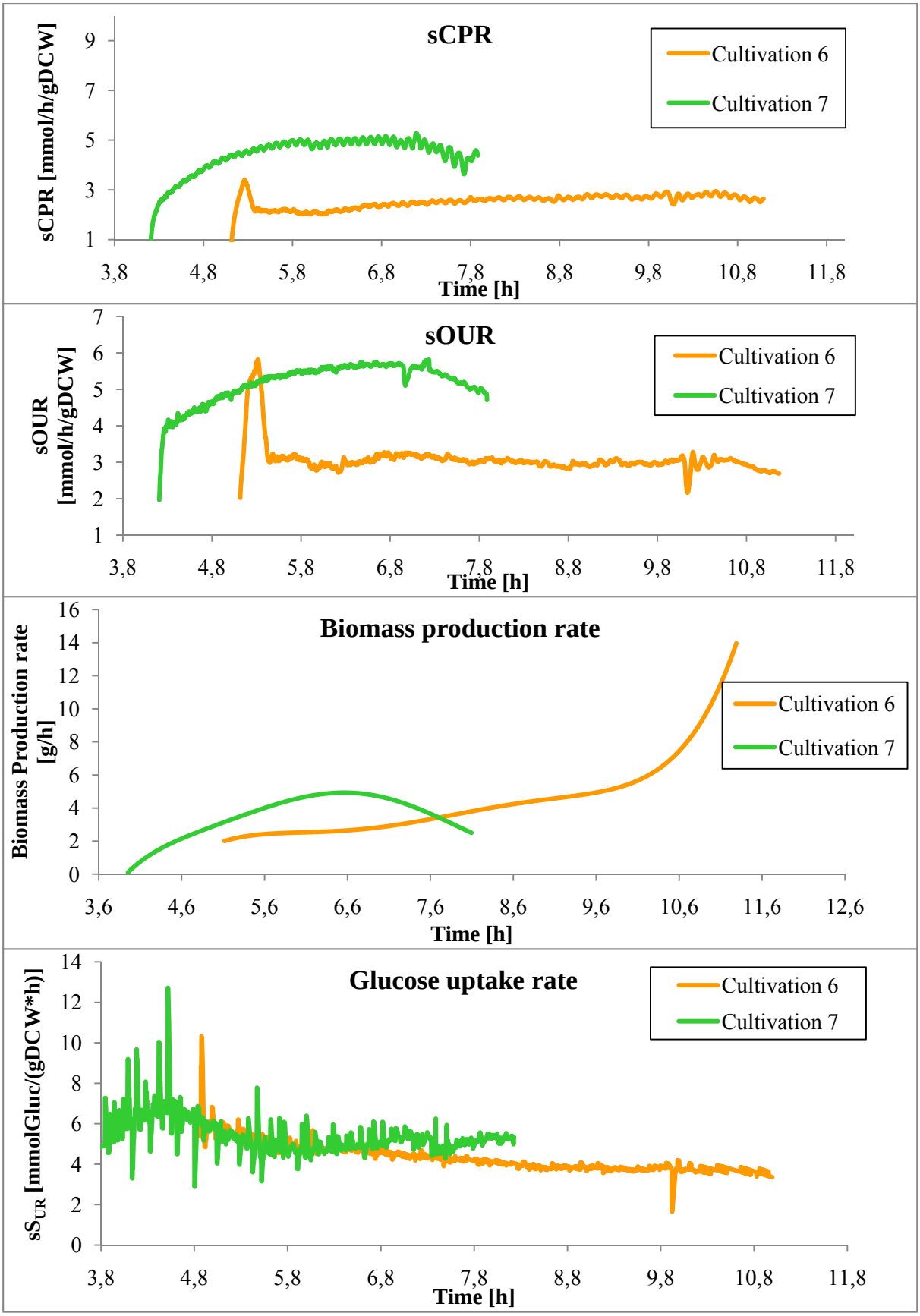

Figure 41: Comparison of specific CPR, specific OTR, specific glucose uptake rate and biomass production rate at different glucose set points.

But cultivation 7 is a short cultivation which represents only four hours of the feeding phase. Here also the specific CPR, specific OTR are increasing gradually in the beginning indicating a normal growth of the cells, but toward the end of the control phase it shows a tendency to decrease. This decreasing tendency may also be due to the metabolic change indicated by the formation of acetate, even though it is not so high. Since there is no oxygen limitation inside the bioreactor, the metabolic change might be an overflow metabolism.

In order to study efficiency of the cultivation process, a carbon balance is made. The result of the carbon balance is given in Table 12.

Table 12: Carbon balance data of cultivations with Kalman filter control

Cultivation	Carbon content of the biomass [%]	Carbon taken in [g]	Carbon coming out [g]	Ratio [C_{out}/C_{in}]
C6	42.77	18.8	18.0	0.96
C7		11.5	10.0	0.87

From the data given in Table 12, the most efficient cultivation process is cultivation 6 which has been carried out at a glucose set point of 0.05 g/L. The carbon flux flow is given in Table 13.

Table 13: Carbon flux distribution in fed-batch cultivations of *E. coli* K1 with Kalman filter control

Cultivation No	Biomass [%]	CO_2 [%]	Acetate [%]
C6	70.67	29.33	0
C7	74.09	24.01	1.6

In cultivation 6, no acetate is obtained and 70.67 % of carbon flux is flowing to biomass. In cultivation 7, the carbon flux to biomass is higher than that of cultivation 6, but both the process cannot be compared simply because cultivation 7 represents only a short period of

bacterial growth in the beginning of the feeding phase. The flux distribution is represented diagrammatically in Figure 42.

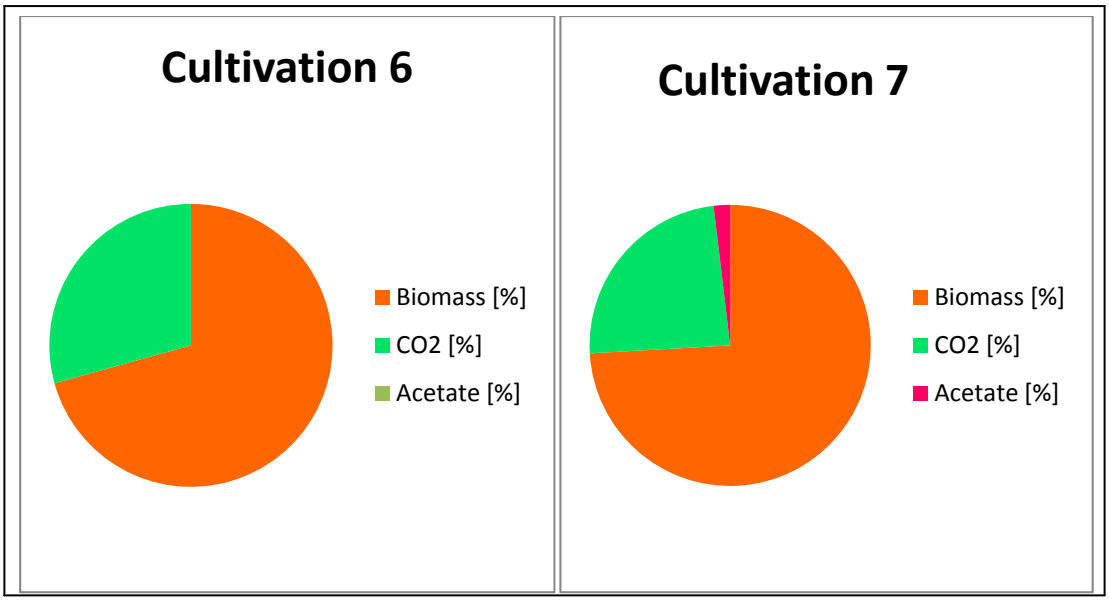

Figure 42: Carbon flux flow in *E. coli* cultivations with EKF control

However, it is clear that a lower glucose set point is favourable for the production of polysialic acid by *E. coli* K1.

4.1.4. Effect of growth rate on the production of biomass and polysialic acid

The cultivation process of *E. coli* K1 has been performed under different cultivation conditions, as batch, glucose limited fed-batch with exponential feeding and fed-batch with EKF based control. It is evident from those experiments that the biomass production is dependent on the growth rate. The growth rate and the yield of both biomass and polysialic acid in different cultivations are presented in Table 14.

Table 14: Comparison of yield factors during the cultivation of *E. coli* at different growth rates

Cultivation Type	Exponential Feeding			EKF control		Batch	
Cultivation No	C3	C4	C5	C6	C7	C1	C2
Growth rate [h^{-1}]	0.11	0.19	0.32	0.37	0.44	0.67	0.75
Biomass Yield [g/g]	0.33	0.40	0.36	0.52	0.47	0.37	0.46
PSA yield [g/g]	0.052	0.031	0.043	0.061	0.046	0.019	0.026
PSA production rate [g/L/h]	0.1	0.1	0.26	0.18	0.14	0.040	0.028

The PSA production rate is calculated as $\Delta PSA/\Delta t$, where Δt is the difference between the initial and final time of the control phase. In Table 14 it is clear that in two batch cultivations, the growth rate is maximum. Cultivation with 0.67 h^{-1} growth rate has an initial glucose concentration of 19 g/L whereas in the other one with growth rate 0.75 h^{-1} has only 5 g/L glucose concentration. The yield of both biomass and PSA are higher when the glucose concentration is 5 g/L. But the production rate of PSA is extremely lower, under this condition.

Under fed-batch conditions with exponential feeding, the biomass is not that much different at the three different growth rates, 0.11 h^{-1}, 0.19 h^{-1} and 0.32 h^{-1}. It is observed that the biomass yield is not showing a large difference on changing the growth rate. But the PSA yield is found to be highest among the three cultivations when the growth rate is 0.11 h^{-1}. It might be possible that the bacteria can integrate more PSA to the cell wall under extremely lower growth rates. But the production rate of PSA is very less and not agreeable in this cultivation.

In cultivations with Kalman based control, the glucose concentration was kept constant at a desired level. In this work, the maximum amount of PSA is obtained in cultivation 6 when

the glucose concentration was minimum i.e., at 0.05 g/L. At this point the growth rate was 0.37 h^{-1}. As the growth rate increases, *E. coli* starts to produce acetate and the biomass yield started to decrease.

Thus it can be concluded that, biomass yield increases with growth rate, reaches a maximum and then decreases. PSA concentration also follows the same pattern. In the cultivations mentioned above, the maximum biomass and PSA production is obtained at growth rate 0.37 h^{-1}. It is obtained by Kalman based control system. Under this cultivation condition no acetate is also formed. In Figure 43, PSA yield and biomass yield are plotted against growth rate.

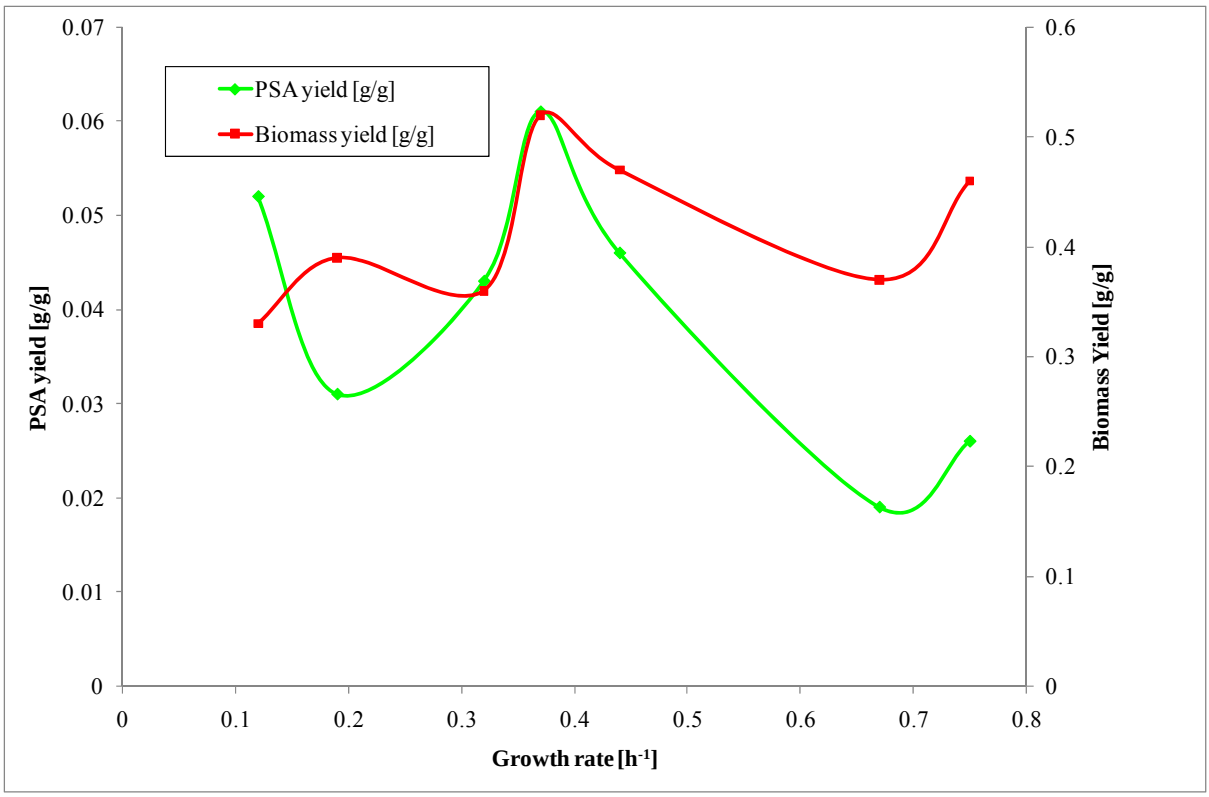

Figure 43: PSA and biomass yield at different growth rates

In Figure 43, the maximum biomass and PSA yield are obtained at growth rate 0.37 h^{-1}. But a higher PSA yield is obtained at 0.11 h^{-1} also. But the PSA production rate is only half of that obtained at growth rate 0.37 h^{-1}. So it cannot be accepted as an ideal condition. But at 0.37 h^{-1} the yield factor and production rate, both are promising and therefore it is inferred that it is the best suitable condition for maximisation of biomass production.

Thus it can be concluded that the optimum growth rate for maximization of biomass production is 0.37 h^{-1} which is achieved through EKF controlled fed-batch cultivation. The controller was able to keep the glucose set point at 0.05 g/L throughout the cultivation so that

maximum growth rate remains the same till the end of the cultivation. The uptake and production rate evaluation points out that the bacteria can remain in a stable oxidative metabolic state under this cultivation condition. Fed-batch cultivation at 0.37 h^{-1} growth rate gives a higher biomass as well as PSA yield compared to the batch cultivation with 19 g initial glucose concentration. Thus, an effective method for the production of PSA is established in this work. At growth rate 0.75 h^{-1}, the PSA again shows an upward deviation. This represents the batch cultivation with initial glucose concentration of 5 g/L. In this cultivation the PSA yield was 0.036 g/g which is higher, but the production rate of PSA is extremely low in this cultivation. Therefore, it is not applicable to economical production purpose.

4.2. Evaluation of fed-batch cultivations of *E. coli* K1 at different glucose set points

EKF controller has been applied during the cultivation of *E. coli* at different glucose set points. The set points of 0.5 g/L, 0.1 g/L and 0.05 g/L are selected in this session for discussion. The objective of these cultivations was to evaluate the performance of the controller at different glucose set point concentrations. Different types of problems came across during different cultivations has also been presented.

4.2.1. Cultivation at 0.5 g/L glucose concentration

In this sub-topic, cultivation of *E. coli* at a glucose set point of 0.5 g/L is discussed in detail. For the sake of convenience, the cultivation is designated as fb1. The set point of 0.5 g/L is comparably higher in this study. The cultivations presented here is not perfect, but portrayed with an aim to discuss the problems came across during the control phase. In Figure 44, the on-line and off-line glucose concentrations of fb1 are presented along with estimated and measured biomass as well as estimated maximal growth rate (μ_{max}).

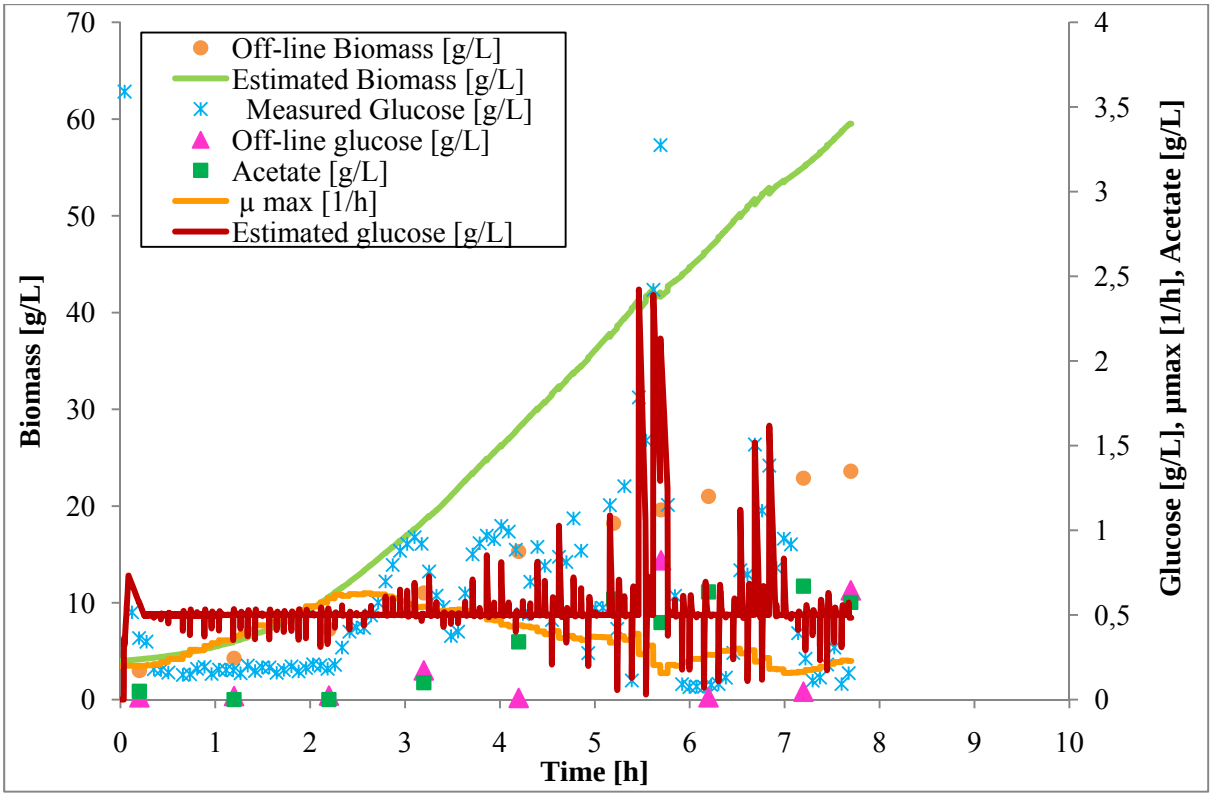

Figure 44: Glucose, acetate, biomass and maximal growth rate during the cultivation of *E.coli* K1 at glucose set point 0.5 g/L

The off-line glucose concentrations are lower than the on-line measured glucose concentrations, indicating that the real glucose concentration inside the bioreactor should not be much higher than the intended set point. Although the offline samples were processed rapidly, a further consumption of glucose by the cells cannot be ruled out.

For the first 2.18 hours, the on-line measured glucose concentration was maintained at an average value of 0.2 g/L whereas the estimated glucose concentration had an average value of 0.51g/L. This might be the consequence of an incorrect initial values assigned to the Kalman Filter. The allocated initial values are recorded in Table 15. The initial value of maximal growth rate (μ_{max}) given to the Kalman filter was 0.2 h^{-1} which is extremely lower for the intended glucose set point. The value of maximal growth rate was increasing continuously for the first 2.2 hours of the feeding phase. Till that point of time, the glucose concentration inside the bioreactor was around 0.25 g/L and as the μ_{max} value reached 0.59 h^{-1}, it came up to the required set point. The peculiar phenomenon of lower glucose concentration during the initial phase might be due to an extremely lower value of maximal growth rate and a higher value of yield factor given to the Kalman filter. The given value of yield factor was 0.5 g/g

whereas the value which is calculated is only 0.28 g/g. Due to these inaccuracies, the volume of the feed solution pumped into the bioreactor might be lower than required. The initial values given to the PI controller was also not suitable enough to compensate this higher difference between the given and original values. Therefore, it took a higher time period to reach the glucose set point concentration. From 2.18 hours to 5.34 hours, the controller was trying to maintain the glucose concentration at the set point, but an oscillatory pattern has been observed in the measurement data. This also supports the fact that the initial values given to the Kalman filter was not fine.

Table 15: Initial parameters given to the Kalman Filter during cultivations fb1

Parameters	**fb1**
Glucose [g/L]	0.5
Biomass [g/L]	4.0
$\mu_{max}[h^{-1}]$	0.2
Volume [L]	1.1

After 5.6 hours, the on-line measured glucose concentration shows abnormally higher values due to the entrapment of air bubbles in the sample flow. Since the initial volume of the culture broth was only 1.1 L, the ceramic membrane which allows the passage of liquid broth for glucose measurement would not be completely immersed into the cultivation medium. Therefore, small air bubbles would come out along with the samples and will got entrapped into the electrode, which gives abnormal measurement data. Thus the FIA system became highly unstable and the measurements were not dependable. The value of maximal growth rate started to decrease from this point onwards due to the instability of the measurement data. The estimated biomass reached the value of 56.5 g/L at the end of the cultivation, whereas the off-line biomass value was only 23.6 g/L. This might be due to the higher value of yield factor given to the Kalman filter.

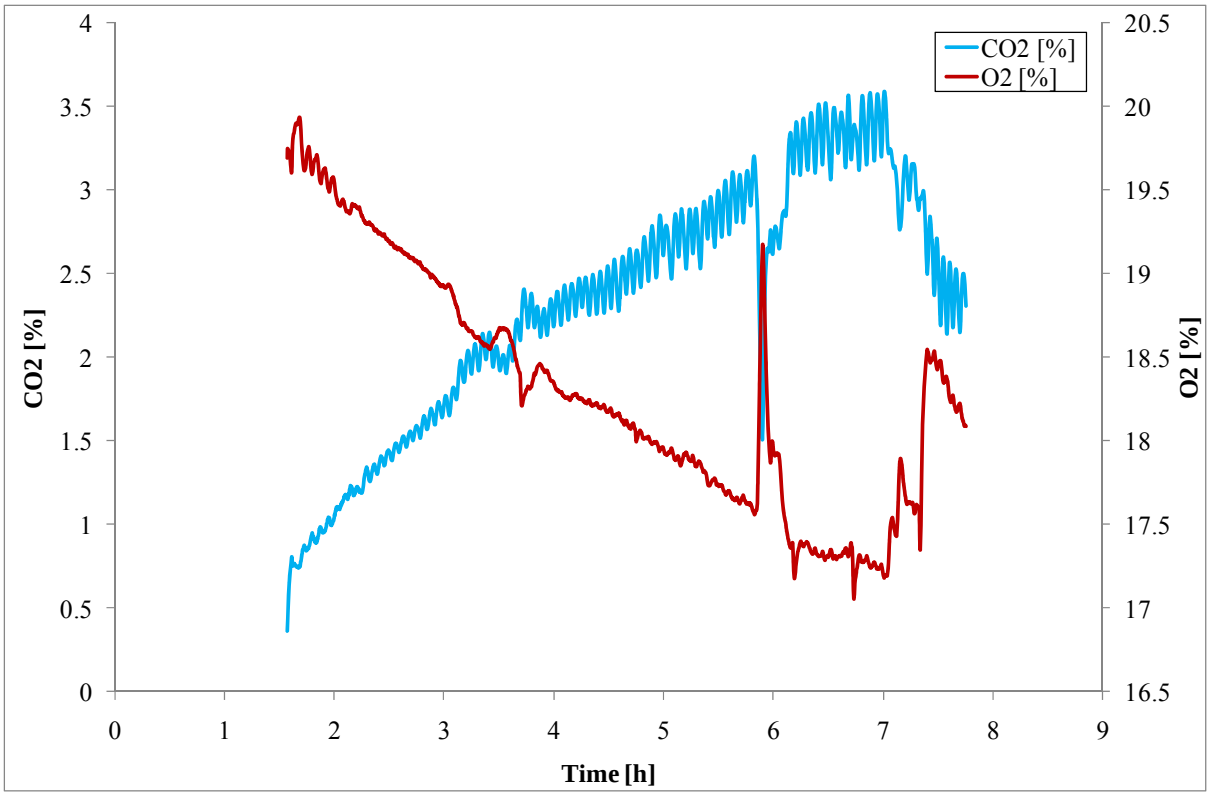

Figure 45: Exhaust gas analysis during the cultivation of E. coli K1 at 0.5 g/L glucose set point concentration.

The exhaust gas analysis data (Figure 45) shows an unlimited growth during the entire cultivation. A large deviation in the graph observed at 5.6 h of cultivation was due to the abnormally higher value of glucose concentration caused by the entrapment of air bubble inside the oxygen electrode. The data from 1.5 h of feeding phase has been presented because of the mal functioning of the RISP.

4.2.2. Cultivations at 0.1 g/L glucose concentration

The cultivations performed at 0.1 g/L glucose set point concentration are discussed under this chapter. For the sake of convenience, these cultivations are designated as fb2 and fb3. The initial values of the control variables given to the Kalman filter in these cultivations are summarized in Table 16.

Figure 46 depicts the EKF control data during the fed-batch cultivation fb2. It can be observed that the on-line glucose concentration shows much higher deviations than expected. This is due to the special condition applied during this cultivation. In this particular cultivation, pure oxygen has been mixed up with air to keep the oxygen level inside the culture medium. But the system could not maintain the dissolved oxygen concentration at a

constant level. It was showing oscillatory behaviour throughout the control phase, which means that the measured glucose concentration is not exact. This is because of the fact that the glucose is measured as the oxygen content of the culture broth. Since the dissolved oxygen concentration inside the cultivation medium is changing frequently, the glucose concentration measured by the oxygen electrode also will be varying.

Table 16: Initial parameters given to Kalman filter during fb2 and fb3

Parameters	**fb2**	**fb3**
Glucose [g/L]	3.9	0.287
Biomass [g/L]	3.0	0.994
$\mu_{max}[h^{-1}]$	0.25	0.4
Volume [L]	1.7	1.7

The average value of the on-line glucose concentration during the feeding phase was 0.186 due to the abnormally higher glucose measurement during the changing of oxygen flow rate.

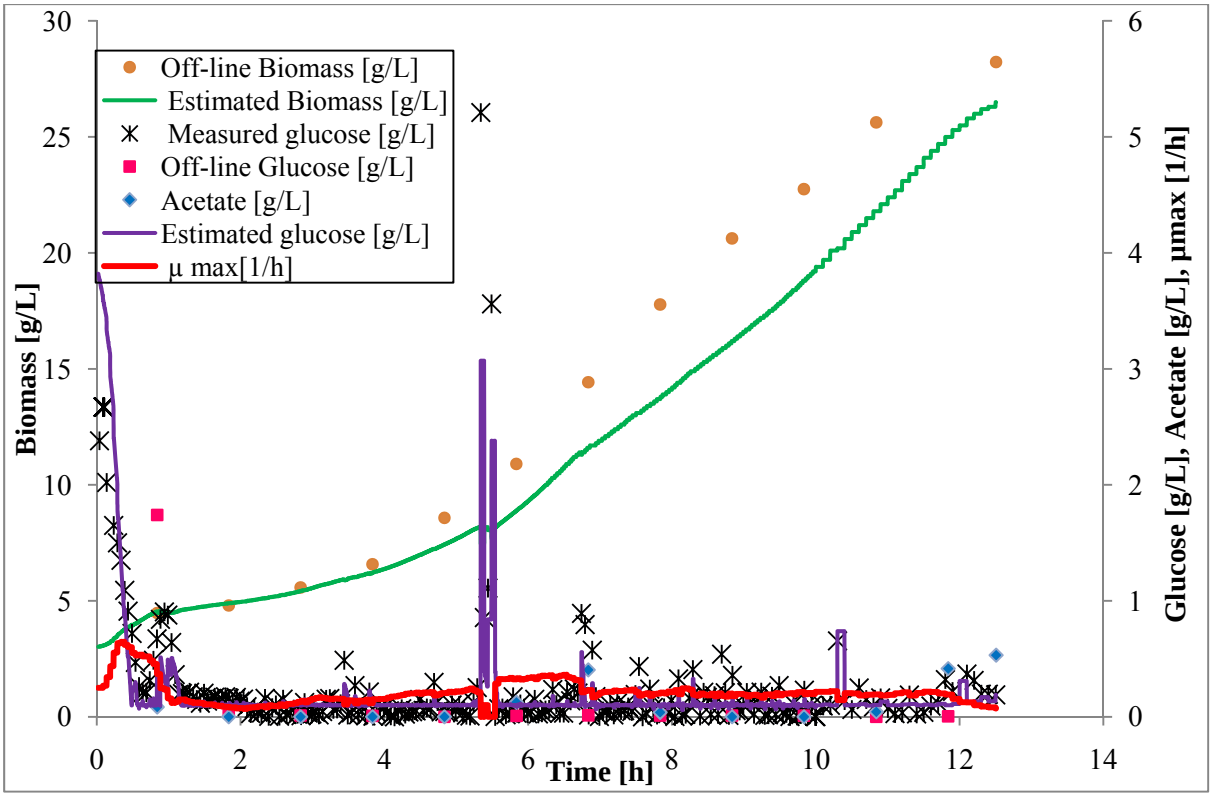

Figure 46: EKF data during cultivation fb2 at glucose set point 0.1 g/L

The highest deviation from the desired set point was observed at 5.34 h of cultivation with a value of 5.21 g/L. This might be due to the addition of antifoam followed by the increment in oxygen flow rate. The problem of air bubble also has been observed in this cultivation. The peaks obtained at 6.74 h as well as 10.3 h are due to the disturbance happened to the system due to air bubble entrapment inside the electrode. The estimated and measured biomass concentration is in good agreement during the initial stage of the control phase, but the measured biomass shows a positive deviation from the estimated value in the later stages even though it is not too much. This might be due to the lower value of yield factor given to the Kalman filter. The value assigned to Kalman filter was 0.25 g/g whereas the calculated yield is 0.31 g/g. The quick response of the Kalman filter to the variation in cultivation system is well evident in this cultivation. As stated above, at 5.34 h of cultivation, the glucose concentration showed a shoot up. The Kalman filter suddenly recognizes the situation and adjusted itself by decreasing the value of maximal growth rate as well as estimated biomass (Figure 46). The maximum acetate concentration obtained during this cultivation was 0.53 g/L which is obtained at the end of the cultivation.

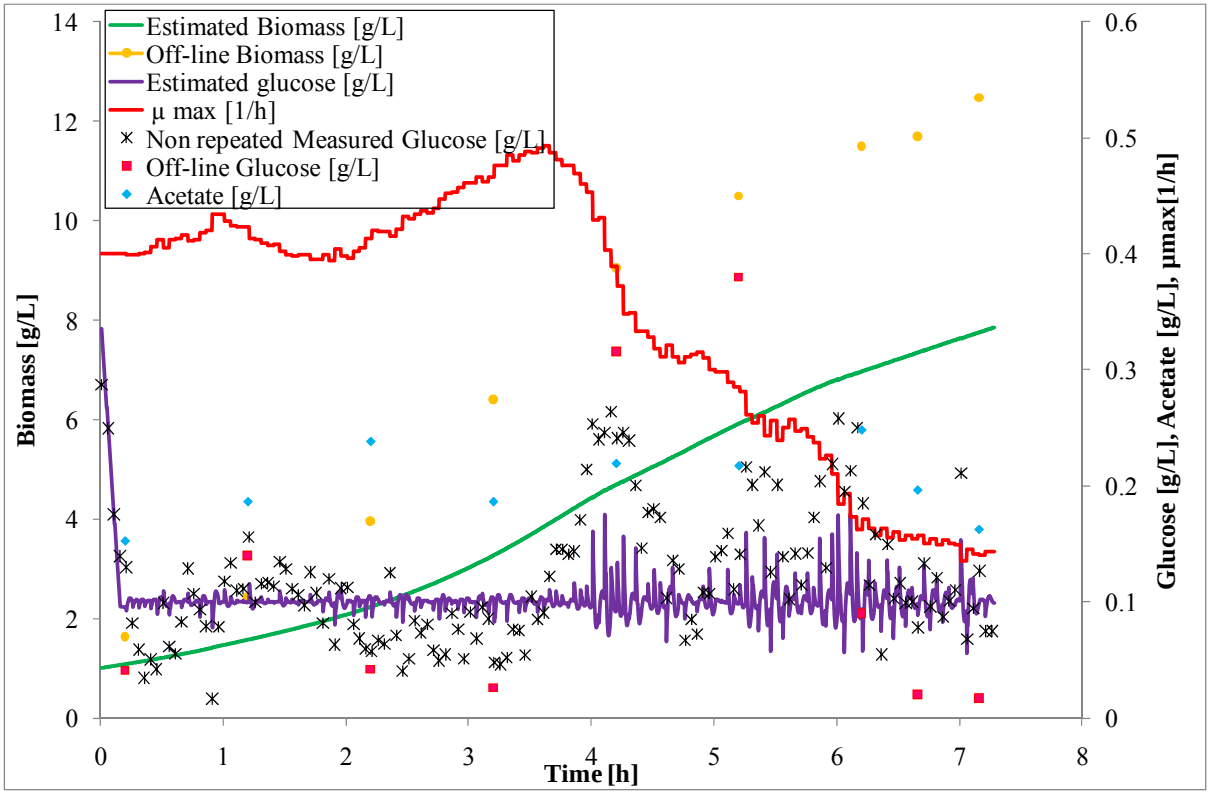

Figure 47: EKF data during cultivation fb3 at 0.1 glucose set point concentration

Since the flow rate of pure oxygen was varying, the calculation of CPR and OTR becomes a tedious process. However, this cultivation data shows that the introduction of pure oxygen to the cultivation system is not so advantageous to the control system.

In fb3 also the glucose set point concentration was 0.1 g/L. In this cultivation the initial biomass concentration was kept lower to increase the length of the control phase without the introduction of pure oxygen. As illustrated in Figure 47, the controller was able to keep the glucose concentration around the set point till 3.8 h of cultivation with an average value of 0.097 g/L. After that point of time, pure oxygen was mixed with air to keep the dissolved oxygen concentration at 30%. The immediate change in oxygen concentration in the air current caused an imbalance of the controller and the glucose concentration was showing a tendency to be higher than the set point concentration. However, the average glucose concentration during the entire cultivation was 0.12 g/L. This change in glucose concentration after the introduction of pure oxygen was accompanied by a decrease in the maximal growth rate. The value of μ_{max} started to decrease from the average value of 0.43 h^{-1} to a value of 0.14 h^{-1} at the end of the cultivation. The value of growth rate calculated from off-line glucose concentration also decreases from 0.43 h^{-1} to 0.29 h^{-1}. The possible reason for this observation might be the withdrawal of 100 mL of cultivation medium from the bioreactor at 9h of cultivation as well as the instability of the measurement system due to the introduction of pure oxygen.

As the cultivation time increases, the difference between estimated and measured biomass increases. This might be caused by the lower value of yield factor given to the Kalman filter. The given value was 0.25 g/g whereas the calculated value was 0.31 g/g. Also the decrease in calculated growth rate is not as sharp as the one observed in Kalman filter. The immediate response of the EKF to the varying environment during cultivation process is evident in this cultivation also. As the measured glucose concentration started to increase beyond the limit of 0.2 g/L, the feeding rate suddenly went down to compensate the immediate effect (Figure 48). The exhaust gas analysis data shows that after the introduction of pure oxygen, the pattern of CO_2 as well as O_2 evolution got disturbed and the carbon dioxide evolution shows a decreasing pattern due to decreased growth rate. The maximum amount of acetate observed in this cultivation was 0.25 g/L which is not too much to inhibit the growth of the organism.

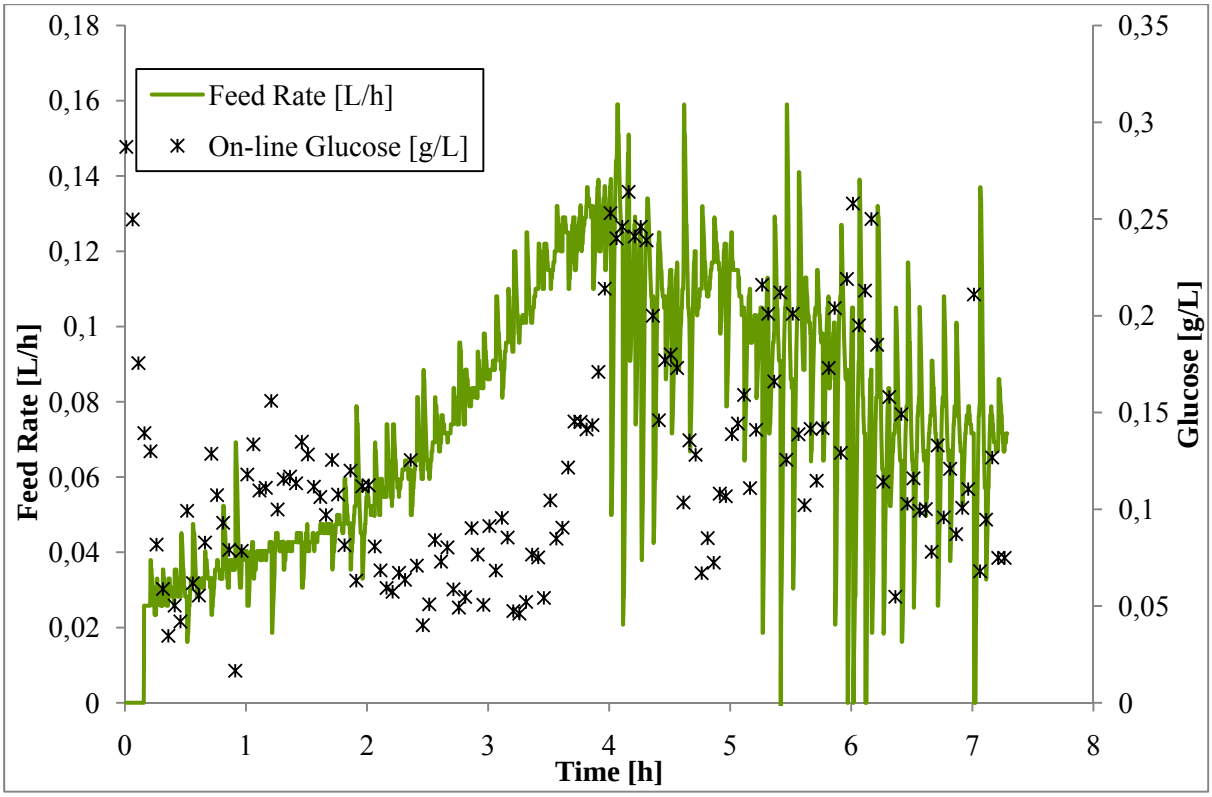

Figure 48: Feed rate and measured glucose concentration during cultivation fb3.

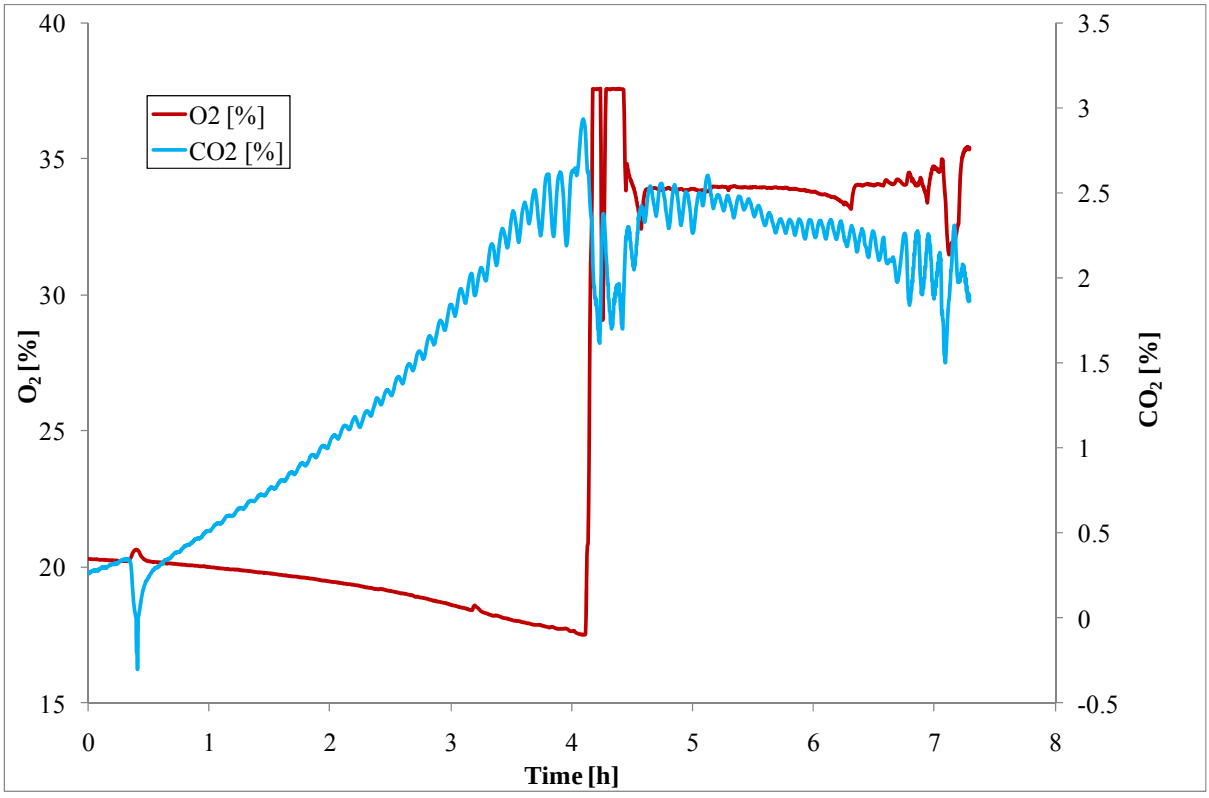

Figure 49: Exhaust gas analysis during the cultivation fb3 at 0.1 g/L glucose set point concentration

4.2.3. Cultivation at 0.05 g/L glucose concentration

A cultivation carried out at a glucose set point concentration of 0.05 g/L is explained in this chapter. Here, the cultivation data till 10th hour of fed-batch phase is illustrated. The initial parameters given to the Kalman Filter is presented in Table 17.

Table 17: Initial parameters given to Kalman filter during fb4

Parameters	**Fb4**
Glucose [g/L]	0.632
Biomass [g/L]	1.005
$\mu_{max}[h^{-1}]$	0.25
Volume [L]	1.5

Figure 50 shows the measured data and the values estimated by the Kalman filter. The mean value of the measured glucose concentration is 0.05 g/L which is in good agreement with the desired set point.

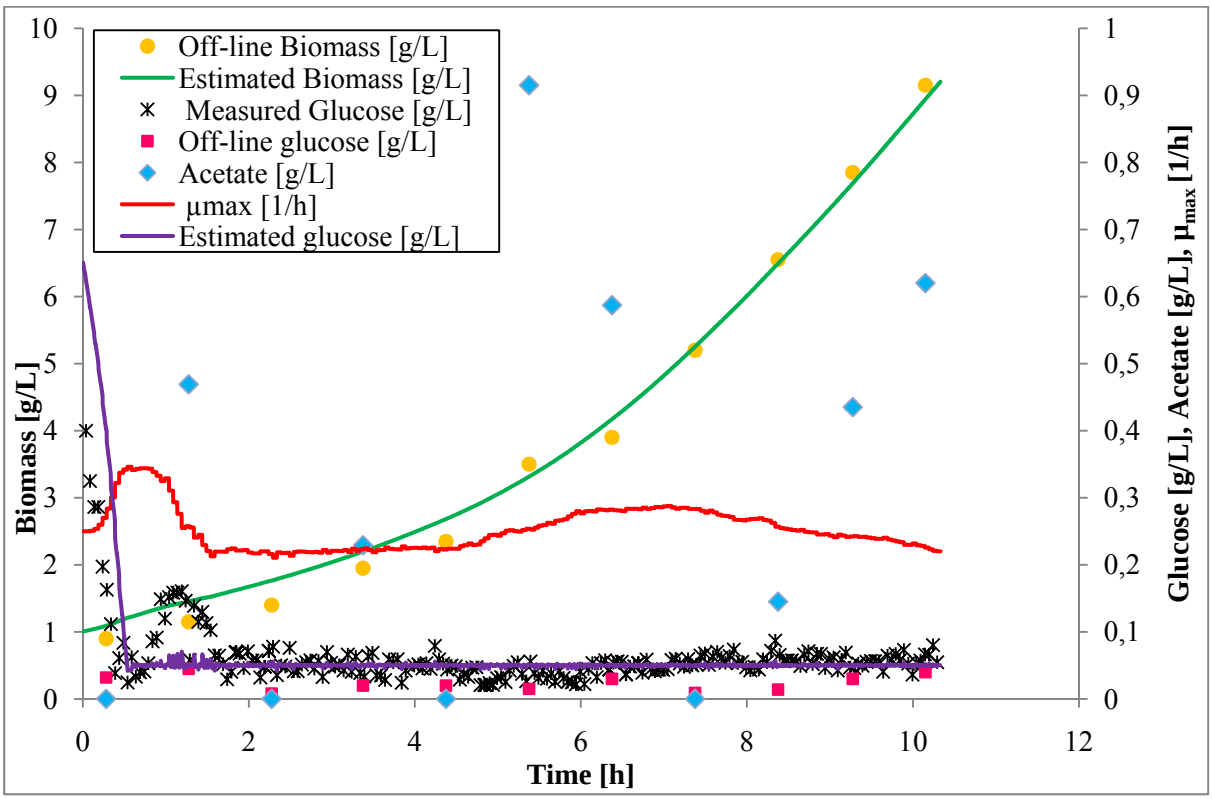

Figure 50: EKF data during fed-batch cultivation fb4 at 0.05 g/L glucose concentration

The controller was able to keep the glucose concentration sticking to the desired set point. A peak in the glucose concentration has been observed in the beginning of the feeding phase (Figure 50). As the batch phase come to the end, the growth rate will be increasing very fast. But when the glucose concentration reaches the desired set point, the available glucose concentration is very less and the bacteria should adjust itself to the new environment. At very low glucose concentration, the growth rate will be lower. Therefore, an additional amount of glucose will be accumulated in the beginning of the feeding phase, till the growth rate and oxygen level got stabilized. At the end of the batch phase the growth rate reached a value of 0. 34 h^{-1}, but when the feeding started the growth rate reduced to 0.23 h^{-1}, then remains almost constant till 4.6 hours and then slightly varied up to 0.29 h^{-1}. The evolution of the biomass concentration calculated by the Kalman filter coincides with the off-line measurements in the later stages of feeding phase although it has a slight deviation from the estimated values in the beginning.

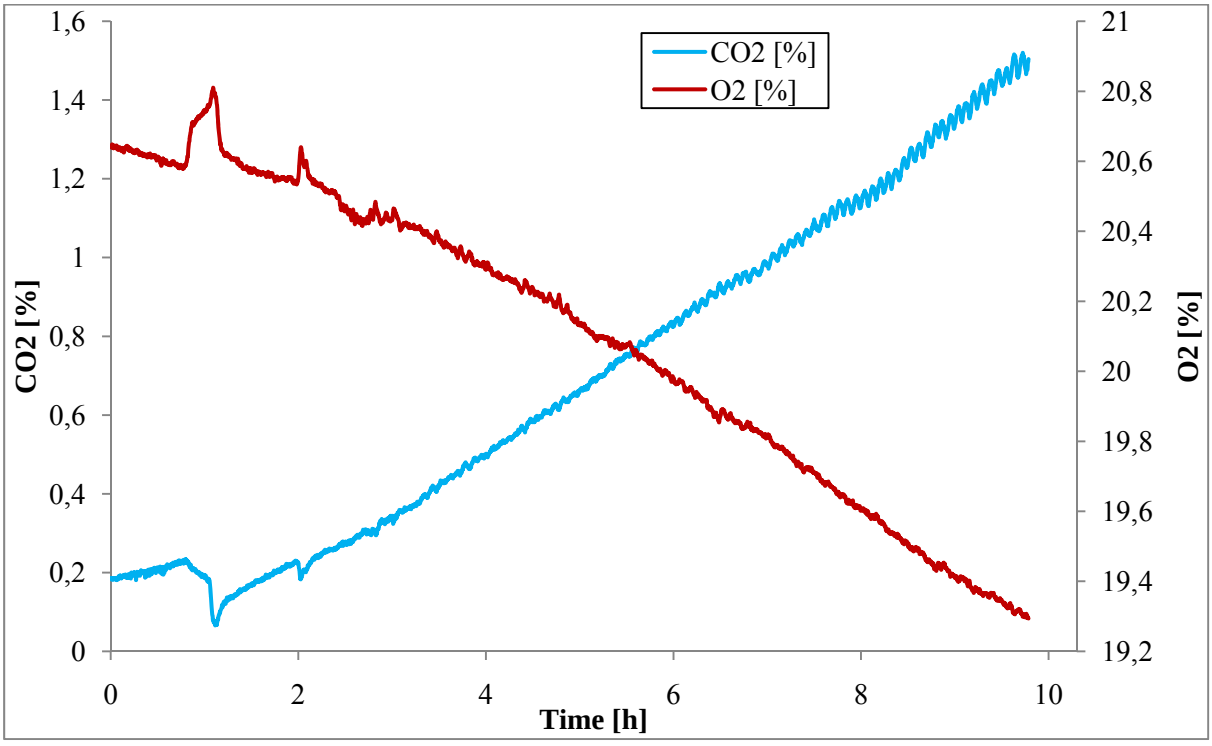

Figure 51: Exhaust gas analysis data during fb4 at 0.05 g/L glucose concentration.

The main problem encountered in this particular cultivation was that even when the glucose concentration was kept at 0.05 g/L, a significant amount of acetate has been observed. At 5.38 h of fed-batch, the acetate concentration reached a value of 0.9 g/L, which was beyond expectation. The distracted pattern of the acetate concentration indicates that there might be

some problem with the GC column used for acetate detection. Another possible reason might be that the offline samples were not properly cooled before handling it for off-line measurements.

As the concentration of oxygen and carbon dioxide in the exhaust gas are important indicators for the growth, metabolism and stability, it can be seen in Figure 41 that except for some minor disturbances, the concentrations evolve steadily in the course of the feeding phase.

5. Summary and conclusion

Polysialic acid (PSA) found wide range of application in tissue engineering. Even if it is highly demanded compound in the scientific world, an effective method for the optimal production of polySia has not been established. On this back ground, the objective of this work was to optimise the production process of polysialic acid through maximization of the biomass production rate by cultivation of *E. coli* K1 2032.

This objective can be achieved through batch and fed-batch cultivations. Fed-batch cultivations have been carried out in two methods; fed-batch cultivations in glucose limited conditions with exponential feeding and fed-batch cultivations at controlled glucose set points using EKF based feed forward/feedback control. Detailed study of the production process of biomass as well as polysialic acid has been carried out under these three cultivation methods.

In all the cultivations, whatever the method employed, a common fact that one come across is the pattern of PSA and biomass production. In all the cultivations the coefficient of PSA to biomass was higher in the starting of the cultivation and gradually it decreases. This might be the effect of complex medium used in the pre-culture. Yeast extract is used in all the pre-cultures and in it the bacteria can grow really well. This means that in complex medium, *E. coli* K1 cells can maintain a higher PSA to biomass coefficient. After inoculation into the mineral medium inside the bioreactor, this effect will be retained for a few more hours, but gradually decreasing. Thus higher PSA to biomass coefficient will be maintained as long as the effect of yeast extract remains in the cultivation media.

Batch cultivations were performed with higher as well as lower initial glucose concentrations. When the initial glucose concentration was 19 g/L, the growth rate was 0.67 h^{-1} with a biomass yield of 0.37 g/g and PSA yield of 0.019 g/g whereas in batch cultivation with 5 g/L glucose start concentration, the growth rate was even higher, 0.75 h^{-1} with a higher biomass yield of 0.46 g/g and PSA yield of 0.027 g/g. In high initial glucose batch cultivation, an oxygen limitation occurred inside the bioreactor accompanied by the production of higher amount of acetate. This limitation might be a reason for lower yield in this cultivation. Another notable factor is the effect of complex media from the pre-culture. The effect of yeast extract used in the pre-culture, will be having its influence for a few more hours in the cultivation and the batch cultivation with 5 g/L initial glucose concentration being a shorter

cultivation than the other batch, will get more affected by it. That might be a reason for higher biomass and PSA yield in this cultivation.

In glucose limited fed-batch cultivations with exponential feeding strategy, growth rate was maintained at a lower level. Three cultivations with growth rates 0.15 h^{-1}, 0.25 h^{-1} and 0.35 h^{-1} were carried out to study the effect of glucose limited growth and lower growth rate on the production of biomass. The biomass yield was more or less the same under these three different growth rates. It is beyond expectations. But the PSA yield shows a higher value at growth rate 0.15 h^{-1}. This indicates the higher PSA integration into the biomass with very low growth rate. But the PSA production rate is extremely lower at very low growth rate.

The third cultivation strategy used in this study was controlling the glucose concentration at a constant value inside the bioreactor using a Kalman based feed forward/feedback controller. Using this controller system two cultivations were performed with glucose set point concentrations of 0.05 g/L and 0.1 g/L. The corresponding growth rates were 0.37 h^{-1} and 0.44 h^{-1}. The comparison of yield factors during these three cultivations points out that the yield of both biomass and PSA was maximum when the glucose set point was minimum. As the set point concentration increases the yield decreases. The uptake and production rate analysis shows that at lower glucose set points, the *E. coli* cells remains in the stable oxidative metabolic state throughout the cultivation so that no undesirable by products such as acetate is observed. So the effective utilisation of the carbon flux for biomass building up is possible under minimum glucose set point concentration. But as the set point increases, the growth rate also increases and the cells will transform from a pure oxidative metabolic state to overflow metabolism associated with production of acetate. This affects the yield factor. Carbon flux will then be directed towards acetate and carbon dioxide than to biomass and the biomass yield will be lower.

Evaluation of all the cultivations on the basis of growth rate and biomass production reveals that as the growth rate increases, the biomass and PSA yield increases, reaches a maximum and then decreases. At growth rate 0.75 h^{-1} a slightly higher yield is obtained. It belongs to the batch cultivation with 5 g/L initial glucose concentration. The higher PSA yield at growth rate at growth rate 0.15 h^{-1} is also not acceptable because the production rate is not satisfactory in terms of time consumption. But in industrial production process and also in process optimisation process, not only the yield factor, but also the product formation rate might be considered. In batch cultivation, the PSA production rate was extremely lower, 0.04

g/L/h compared to the one at growth rate 0.37 h^{-1}, i.e., 0.18 g/L/h. Considering both these important factors together, the fed-batch cultivation at a growth rate of 0.37 h^{-1} is more effective in biomass and in turn the PSA production.

The biomass-PSA concentration plot of all the cultivations shows that as the biomass concentration increases, the PSA concentration also increases. In all the cases a linear relationship exists between biomass and PSA concentration, but it varies depending on the growth rate indicating difference in the metabolism of the cells.

Thus an EKF based bioprocess control system has been established for controlling the glucose concentration inside the bioreactor at a lower level during the cultivation of *E. coli* K1 for the production of polysialic acid and is proved to be most effective in maximizing the production of biomass and thereby the yield of PSA.

EKF based control system has been used to maintain the glucose level inside the bioreactor at a desired level. Since it is provided with feedforward/feedback control system, it can effectively handle the critical variations happening inside the bioreactor. But the control system works based on the glucose measurements carried out by highly sensitive FIA system. Therefore, even minute disturbances can make large variations in control process. So caution must be taken to avoid disturbances to the FIA system. The volume of the cultivation medium is very important because the ceramic membrane which allows the passage of sample for on-line glucose measurements should be immersed completely inside the medium so that the air bubble can be avoided. If air bubble even with very small size enters into the sample stream, it will stick to the membrane of the oxygen electrode which will affect the glucose measurement and finally result in imbalance of the entire measurements. Another critical factor is the selection of initial parameters given to the Kalman filter. If there is much difference between the assigned value to the Kalman filter and the original value inside the bioreactor, it will affect the control by taking longer time to adjust the parameters to the real value inside. But small and instant variations will be successfully handled by the Kalman filter.

One of the effective methods for increasing the feeding phase during the cultivation is to maintain the dissolved oxygen level above 30% by mixing pure oxygen with air. But this sudden variation in the flow rate and oxygen concentration will adversely affect the glucose measurements because the glucose concentration is measured as oxygen by the FIA system.

Therefore, if the dissolved oxygen concentration is not maintained at a constant level, then the measurements may not be dependable.

The method developed in this work is applicable only for the production of PSA in laboratory scale. It is desirable to scale up the process to obtain large scale production. Since the work tension for keeping the glucose concentration steadily and reliable for longer control phase is too much especially at higher biomass concentrations, it is desirable to upgrade and modify the FIA system for online glucose detection before attempting the scaling up process.

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7. Abbreviations

ADP	Adenosin diphosphate
ATP	Adenosin triphosphate
CAFCA	Computer Assisted Flow Control and Analysis
CO_2	Carbon dioxide
$CO_{2,\ Start}$	Carbon dioxide concentration at the reactor inlet
Co-A	Coenzyme A
CSTR	Continuous stirrer tank reactor
CV	Coefficient of variation
DCU	Digital Control Unit
DCW	Dry cell weight
DCW_{PR}	Biomass Production Rate
E{}	Expectation value from {}
E. coli	*Escherichia coli*
EKF	Extended Kalman Filter
e(k)	Deviation from the set point at time point $t = kT_A$
$e(\tau)$	Control error
f	Function describing the change of the system state variable with the time
FAD	Flavin adenine dinucleotide
FIA	Flow Injection Analysis
$F(t)$	Jacobean matrix of $f(x(t), u(t), t)$
GOD	Glucoseoxidase
H_2O	Water
H (t_i)	Jacobean matrix from $h(x(t_i))$
$h(\hat{x}(t_i), t_i)$	Expected measurement value calculated by the estimated values of the state variable before filtering

K	Amplification factor
KCl	Potassium chloride
K_m	Monod Saturation Constant
$K(t_i)$	Kalman filter gain
LB	Luria Broth
M_S	Molar mass
NAD	Nicotinamide adenine dinucleotide
OD	Optical Density
ORK	Ordinary Runga Kutta
$O_{2,Start}$	Oxygen concentration at reactor inlet
P	Estimation error covariance matrix
PID	Proportional Integral Derivative
$P_{new}(t_i)$	New estimation error covariance
polySia	Polysialic acid
PSA	Polysialic acid
Q	Spectral density matrix of process noise
R	Measurement error covariance
r_X	Rate of biomass formation
r_S	Rate of substrate consumption
$sCPR$	Specific carbon dioxide production rate
$sOTR$	Specific oxygen uptake rate
S_t	Substrate concentration at time t
S_0	Substrate concentration in the feed solution
S_{SP}	Substrate Set Point Concentration
sS_{UR}	Specific substrate uptake rate

$S(t_i)$	Measurement noise
TCA	Tricarboxylic acid
T_A	Time interval between two measurements or sampling time
T_D	Derivative time constant
T_I	Integration time constant
$u(k)$	Control Value
$u(t)$	Input variable
$V(t)$	The reactor volume at time *t*
$\dot{V}(t)_{Feed}$	Feed rate at time *t*
$\dot{V}_{Sample}$	On-line sample flow rate
w(t)	Set point
$x_j(t)$	Real value of the state variable
$\hat{x}_j(t)$	Estimated value of the state variable
$\hat{X}_{new}(t_i)$	Filtered values of the estimated state variable
$\hat{X}(t_i)$	Estimated value of the state variable before filtering
X_t	Biomass at time t
x(t)	Output variable
$x(t_i)$	System variable at discrete time step
$x_w(t)$	Control deviation
Y	Yield coefficient
y (t)	Correction variable
$y(t_i)$	Measurement value at time step t_i
$z(t)$	Process noise
μ	Specific growth rate
μ_{max}	Maximal growth rate

8. List of Tables

9. List of figures

10. Appendix

A.1. Composition of the Feed Solution for *E.coli* K1

Substance	Concentration
Glucose	100 g/L
Ammonium Sulphate	27.5 g/L
Mineral Solution	2ml/L
Calcium Chloride	1.76ml/L

A.2. Composition of FIA buffer

Substance	Concentration
K_2HPO_4	4.3 g/L
NaH_2PO_4	2.1 g/L
NaCl	3.0 g/L
EDTA	1.5 g/L
NaN_3	0.065 g/L

- To avoid froth formation, a few drops of Triton-X are added [Maximum 1 drop per litre]
- The pH must be 6.8
- Sodium nitride is poisonous.

A.3. Calcium Chloride Solution for *E.coli* K1 2032

Substance	Concentration
$CaCl_2.2H_2O$	14.7g/L

A.4. Reactor Medium for *E. coli* K1 cultivation

Reactor Medium	
NaCl	1.2g/L
K_2SO_4	1.1g/L
Mineral Solution	1ml/L
$CaCl_2$ Solution	0.013g/L
Nitrogen Source	
$(NH_4)_2SO_4$	10g/L
Buffer	
K_2HPO_4	6.665g/L
KH_2PO_4	0.25g/L
Substrate	
Glucose	5 g/L

A.5. Mineral Solution for *E.coli* K1 cultivations

Minerals	**Concentration**
$MgSO_4.7H_2O$	150g/L
$FeSO_4.7H_2O$	1g/L
$CuSO_4.5H_2O$	1g/L

A.6. Pre-culture Medium for *E. coli* K1

Substance	**Concentration**
Bacto-Tryptone	10 g/L
Bacto yeast extract	5 g/L
NaCl	10 g/L

11. Bio-data

Personal Data:

Date of Birth	10th May 1980
Place of Birth	Kerala / India
Marital Status	Married
Nationality	Indian

Academic Data:

Ph.D.	Since 2006 working in the research group of Prof. Dr. Bernd Hitzmann in the Institute of Technical Chemistry, University of Hannover, Germany Theme: Establishing a control system for maximisation of biomass production during fed-batch cultivations of *E. coli* K1
B. Ed.	2003-2004 Physical Science, M.G. University, India
M. Sc.	2000-2002 Polymer Chemistry, M.G. University, India
B. Sc.	1997-2000 Chemistry, M.G. University, India
Pre-degree	1995-1997 Physics, Chemistry, Biology, M. G. University, India

Professional Experience:

Teaching experience	2004-2005 Worked as a lecturer, St. Ann's Higher Secondary School, Chengannur, India
Language skills:	English, German (Basic), Malayalam (Mother tongue), Hindi
Computer skills:	MS office, Origin, Corel Draw, Matlab (Basic)

Posters

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2. Bioperspectives 2008, Hannover, Germany 2008: R. Chen, **J. John**, B. Hitzmann, T. Scheper: Comparison of polysialic acid production during batch and fed-batch cultivations *of Escherichia coli K1*
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