OPTIMISING THE PRODUCTION OF BACTERIAL CELLULOSE IN SURFACE CULTURE

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Contents

1 INTRODUCTION

The biosynthesis of cellulose not only takes place in plants but also in some species of bacteria, algae and fungi. The most efficient microbial producers of cellulose are gram-negative, acetic acid producing bacteria of the genus *Gluconacetobacter xylinus*. Bacterial cellulose produced by these bacteria is one of the most interesting materials of white biotechnology. The scientific and commercial interest in bacterial cellulose nowadays results from its specific product characteristics. Bacterially produced cellulose distinguishes itself from plant cellulose by a high crystallinity and purity, as it is free from biogenic concomitant components and molecular inhomogenities. Equally impressive is the mechanical firmness of humid membranes from bacterial cellulose and their capability to absorb approximately 100 times their dry weight as aqueous solutions. Because of these product characteristics, bacterial cellulose finds applications in many different branches of industry.

1.1 History of the research on bacterial cellulose

In spite of the fact that the syntheses of an extracellular gelatine-like material by *Gluconacetobacter xylinus* was described for the first time in 1886 [1], bacterial cellulose has not received more attention before the second half of the 20th century. Possible nutritive media for *Gluconacetobacter xylinus* and their optimization were described by Hestrin et al. between 1947 and 1954 [2, 3]. A first report on industrial applications was given in 1967 by Lapuz et al. who used bacterial cellulose as food thickener [4]. Marx-Figini and Pion have specified the degree of polymerisation and the molecular weight distribution of bacterial cellulose between 1974 und 1976 [5]. In 1986, Ring et al. used bacterial cellulose as bandaging material for wound dressing [6] for the first time. At the end of the 1980's, Fantana et al. used this polymer successfully in the treatment of burns [7, 8]. Since the early 1990's, besides the research on applications, the activities in research and development were intensified in the field of biotechnological production of bacterial cellulose. The main activities took place in Japan, the USA and Germany.

1.2 Physiological functioning and biosynthesis of bacterial cellulose

The major part of existing bacteria synthesizes extracellular polysaccharides, which form a cover around the cell [9]. Bacterial cellulose is an example for such a substance. The aerobic cellulose producing bacteria immobilize themselves within a matrix of cellulose fibres. This enables them to colonise in the interphase between solid and liquid substrates and air to have optimal access to the carbon substrate of the solid respectively liquid phase and the oxygen of the air [10]. Furthermore, the bacteria immobilised within the cellulose matrix are less sensitive towards changes of the environmental conditions, so fluctuations of moisture or the attendance of competitive bacteria can be tolerated more easily.

The biochemical route to cellulose biosynthesis is integrated in the carbon metabolism of the bacterium and consists of five fundamental enzyme catalysed steps [11, 12]. The biosynthesis takes place between the outer membrane of the cell and the cytoplasm membrane by cellulose synthase, a

membrane-fixed enzyme which is connected with each one at the surface of the bacterium localized pores. About 50–80 of these longitudinal arranged enzyme complexes are situated in one bacterium. The glucose, which is needed for biosynthesis of the bacterial cellulose is transported actively by glucose permease through the cell wall into the inner cell. There, the glucose is inducted into the cell metabolism. A part of the glucose is phosphorylated into glucose-6-phosphate by glucokinase. Phosphoglucomutase which transforms it into glucose-1-phosphate and further to UDP-glucose by UDP-Glc-Phosphorylase [13]. This compound is the substrate for cellulose synthase which as a key-enzyme in this process connects UDP-glucose to the end of the growing polysaccharide chain. This process takes place within the cell membrane. The cellulose thus formed leaves the enzyme complex as elemental fibril. Numerous elemental fibrils aggregate to microfibrils which again turn into macrofibrils. The aggregation of the macrofibrils finally leads to the formation of cellulose fibres.

1.3 Properties and applications of bacterial cellulose

Bacterial cellulose is built out of β-1,4-connected D-glucose. Their repetition unit is β -cellobiose, consisting of two units of anhydroglucose. For characterization of bacterial cellulose, often the degree of polymerisation (DP) is used, which reaches values of up to 12.000 [14]. Numerous hydrogen bonds cause the cellulose chains to join to highly ordered structures. Bacterial cellulose constitutes an elongated, linear, partially crystalline condensation polymer which develops an elemental fibril and microfibril morphology, in which crystalline and amorphous phases alternate. Both, the allocation as well as the amount of crystalline and amorphous phases influence the macroscopical properties of bacterial cellulose [14]. The smallest morphologic unity of bacterial cellulose is constituted by the elemental fibril whose diameter is 2-4 nm [15, 16, 17]. The elemental fibrils aggregate to microfibriles with a diameter of 10–95 nm [18] and further to macrofibrils with a diameter of 60-400 nm [19], which forms the cellulose fibre with a diameter of some µm. Fibres of bacterial cellulose are about 100 times thinner than fibres from plant cellulose. The three-dimensional structure of bacterial cellulose leads to a high elasticity and a high mechanical tensile strength (Tab. 1).

Material	**Young´s Modulus [GPa]**	**Tensile Strength [MPa]**	**Elongation [%]**
Bacterial cellulose	15–35	200–300	1,5-2
Polypropylene	1-1,5	30–40	100-600
Polyethylene Terephthalate	3–4	50–70	50-300
Cellophane	2–3	20–100	15-40

Table 1. *Mechanical properties of Bacterial cellulose compared with other organic membranes*

In contrast to plant cellulose, bacterial cellulose is chemically pure because it is not associated with accompanying substances like hemicellulose, lignin and pectin. Due to its macroscopic structure, bacterial cellulose has a high bonding capacity for hydrophilic substances (up to 99% of their dry

weight). The liquid between the cellulose fibres can be released by pressure. Therefore, even by simple downstream processes, it is possible to achieve a chemically pure material.

The unique properties of bacterial cellulose enable numerous applications to be feasible. At the Research Centre for Medical Technology and Biotechnology at Bad Langensalza, applications for medicine, cosmetics industry and in the field of tissue engineering are focused.

In the past, it could be shown by own (and external) research projects, that wound dressings produced of bacterial cellulose have a positive influence on the healing process and that the material is suitable as a temporary skin-replacement in cases of severe injuries. Mayall et al. used wound dressings made of bacterial cellulose for the treatment of chronic wounds on legs and thus proved that the healing period and the risk of contamination could be reduced significantly [20]. Farah et al. observed anodyne properties and an improved evacuation of wound exudates [21]. Czaja et al. applied wound dressings made of bacterial cellulose on burns and observed a significant reduction of fluid loss and therefore a considerable advancement of the wound healing process [22].

The reason for the acceleration of healing processes by biotechnologically synthesised cellulose can not be convincingly answered yet. According to Czaja et al., these effects are attributed to the 3D-nanostructure of the material. He suggested that wound dressings made of biotechnologically synthesised cellulose could fit into the wound surface up to nanometer dimensions. Therefore, optimal humidity conditions results [23].

It could be proved by the help of experiments on sheep and horses that wound dressings made of bacterial cellulose cause a significant reduction of the wound contraction time. No hyperplastic granulation appears and aseptic and therefore healing-supporting circumstances could be achieved under the wound dressing material [24].

The excellent bio-compatibility of bacterial cellulose was also proved in cell culture experiments. It could be shown that sensitive cells like human osteoblasts, equine osteoblasts, anulus fibrosus (human or animal cells), nucleus pulposus (human or animal cells) and mesenchymal stem cells are optimally cultivable on bacterial cellulose [25].

The Research Centre for Medical Technology and Biotechnology offers membranes made of bacterial cellulose under the trade name “NanoMasque”, containing cosmetic ingredients. Within the framework of professional cosmetic treatments, these membranes are applied on the skin of the face. Herewith, an optimal transition of the cosmetic active ingredients into the skin shell ought to be achieved [24].

Besides the life-science-application, numerous technical applications of bacterial cellulose and applications in food industry have been described. Technical applications vary from speaker’s membranes for highest requirements [26], electronic paper [27], aggregates for specific papers [28] up to membranes for fuel cells [29] and membranes for the separation of mixtures of water and organic dissolvers [30]. In the food industry, bacterial cellulose is mainly used as dietary filling and stabiliser for foams, gels and emulsions [31, 32, 33]. Probably the most famous commercial application of bacterial cellulose is the dessert called “nata de coco“, which is foremost widespread on the Philippine Islands [34].

1.4 Biotechnological production of bacterial cellulose

The biotechnological production of cellulose is possible in both, in submerged fermenters and in surface cultures. The type of cultivation affects the appearance of the final product. On the one hand, granular structures, which are allocated equally within the nutritive medium, are produced during the process of submerse cultivation. On the other hand, a homogeneous and compact cellulose layer is built in the surface culture.

Although there are less applications for granular structures of bacterial cellulose than for bacterial cellulose membranes, the submerged culture was of main interest in the past, because of the easier scale up, an uncomplicated instrumentation and the possible access to available fermentors. In the 1990's, the establishment of two large-scale-production-methods for the production of bacterial cellulose were established. The Imperial Chemical Industry process was either held with agitated fermenters or with airlift-reactors [35], the Weyerhaeuser Process took place in a deep tank agitation fermenter [36].

Membranes from bacterial cellulose is an especially highly attractive raw material with broad range of applications explicitly in the life-science-sector. But regardless of this proven popularity, the development of optimized surface culture methods for the production of membranes made of bacterial cellulose was not developed as much as it should have been according to its importance. The completed studies thus far restrict it to the optimization of the traditional surface culture by advancement of the nutritive media configuration, special methods for the treatment of inoculum and the genetically modification of the used bacteria [37, 38, 39].

The bacterial cellulose formation in surface culture occurs discontinuously in e.g. aquarium-like culture boxes. These boxes are filled with a nutritive medium under sterile conditions after Schramm and Hestrin [40], inoculated with a suspension of *Gluconacetobacter xylinus*, sealed to protect it from external infections and incubated at a temperature of 30°C (no movement). After a dead time of two up to three days a visible cellulose formation starts. The insular shaped fragments become a coherent cellulose layer at the liquid surface. The layer thickness increases and reaches a thickness of 3–4 centimetres within 3–4 weeks. After end of cultivation, this cellulose layer can be divided into membranes of the favoured thickness. However, the traditional surface procedure is not suitable for mass production, particularly because of the limited product yield and the inefficient proportion between the work-intensive preparation steps and the product formation phase.

A suitable process, which allows the production of bacterial cellulose membrane in commercial amounts does not exist yet. The only relevant approach of the past to achieve this purpose were bioreactors, in which inoculated surfaces in the form of rolls [41] and plates [42, 43] rotated in a nutritive medium reservoir and which were covered by bacterial cellulose in the course of the cultivation.

However, there are no reviews which document the transition of these methods from laboratory scale to pilot scale. Our own experiments with a rotating disc reactor showed that the quality of bacterial cellulose produced in that way does not conform with the quality requirements, which are needed for medical or cosmetic applications.

1.5 Motivation and Concept

The fact that membranes made of bacterial cellulose has enormous application potential and that no sustainable solutions for mass production were developed until today, constitute the main motivation for working on this topic.

The basis of this work is given by the traditional surface method, which in general generates cellulose in excellent quality. The thus far published results suggest the conclusion that this process is not exclusively limited by the bio-process that is the microbial conversion from glucose to cellulose. This means that measures which only focus on the optimisation of the bio reaction, e.g. by substrate optimisation and increasing of the microbiological performance, will not results in the fundamental optimisation of the process.

Also in the present study, the central element is given by the bio reaction, characterised by consumption of substrate and growth of bacteria. Besides that, it is also necessary to identify how these processes are effected by external chemical and physical conditions such as substrate supply or product removal.

The mathematical simulation increasingly contributes to the efficient development of biotechnological processes. Therefore, different mathematical models display, explain and advance the experimentally acquired results in this study. On this basis, measures are worked out which lead to an real increase of product yield.

At the end of this research, the development of a new bioreactor for the optimized production of bacterial cellulose, is presented. The results achieved by this bio-reactor provide evidence of this claim.

1.6 Nomenclature

The table contains abbreviations and units used in the text. The symbols used for mathematical models will be at according passages in the text for better understanding.

Chapter 1

approx	Approximately
BC	Bacterial cellulose
DP	Degree of Polymerisation
e.g.	For example
fzmb	Research Centre for Medial Technology and Biotechnology
Glc	Glucose
GPa	Giga pascal
MPa	Mega pascal
nm	Nanometer
Tab.	Table
UDP	Uridindiphosohat
3D	Three dimensional

Chapter 2 (additional to previous chapters)

°C	Degree celsius
µl	Micro litre
ACC_j	Accumulation of a component j
cfu	Colony forming units
d	Diameter
Fig.	Figure
g	Gram
g/l	Gram / litre
GEN_j	Generation of a component j
H_2O	Water
IN_j	Inflow of a component j
M	Molar mass
ml	Milli litre
mol/m^3	Mol / cubic meter
N_2	Nitrogen
Na_2HPO	Sodium-di-hydrogen-phosphate
O_2	Oxygen
OUT_j	Outflow of a component j
pH	Potentia hydrogenii
SH	Schramm-Hestrin (nutrient medium)
t	Time
UV/VIS	Ultraviolet / visual

Chapter 4 (additional to previous chapters)

kg/m^3	Kilogram / cubicmeter
g/d	Gram / day
g	Gram
r^2	Coefficient of determination

Chapter 5 (additional to previous chapters)

bar	Bar
cm^2	Square centimetre
F_{max}	Tensile strength at break
GHz	Gigahertz
KHz	Kilohertz
l/h	Litre / hour
MHz	Megahertz
ml/min	Millilitre / minute
mm/d	Millimetre / day
N	Newton
PC	Personal computer
rpm	Rounds per minute

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2 EVALUATION OF SUBSTRATE MASS TRANSFER INFLUENCES ON THE PRODUCTION OF BACTERIAL CELLULOSE

2.1 Abstract

The interest in cellulose produced by bacteria from surface cultures has increased steadily in recent years because of its potential for use in medicine and cosmetics. Unfortunately, the low yield of the production process has limited the commercial usefulness of bacterial cellulose. This study dealing with the production of bacterial cellulose using (batch) surface culture, firstly present a complete and complex analysis of the overall system, which allows a fundamental optimization of the production process to be performed. This material has many applications but the low yield of the process limits its commercial usefulness. In the following chapter, the effect of the rate of mass transfer of substrate on the microbial process, which is characterized by the growth of the bacteria, product formation, and the utilization of the substrate by the bacteria, is studied. A fundamental model for the diffusion of glucose through the growing cellulose layer is proposed and solved. The model confirmed that the increase in diffusional resistance is indeed significant but other factors will also need to be taken into account.

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2.2 Introduction

Cellulose Production by Gluconacetobacter xylinus

It is known for more than a century that the gram-negative, rod-shaped aerobic bacteria of the strain *Gluconacetobacter xylinus* (formerly called *Acetobacter xylinum*) produce cellulose (bacterial cellulose, BC) when fed with carbohydrates [1]. The bacterial cellulose has been found to have a unique structure, composed of very thin fibres that form an ultrafine network. Cellulose produced by *Gluconacetobacter xylinus* is chemically pure, free of lignin and hemicelluloses contrary to wood celluloses and has a high degree of polymerisation. In nature, cellulose producing bacteria are found in rotten fruits and vegetables with more than thirty cases having been reported [2]. The reason why the microorganisms convert glucose into the high-polymeric cellulose has been a puzzle for biologists. Scientists have suggested that the bacteria immobilize themselves in such a “cage” to maintain their position between a substrate rich fruit surface and the oxygen rich air space. [3, 4, 5]

The biotechnological production of cellulose is possible in both, in submerged fermenters [6] and in surface cultures. Although the chemical structure of cellulose from surface culture is identical with the submerged material, the phenotype of both of these forms are totally different. In agitated or aerated submerged culture, the cellulose accumulates in the form of pellets. However, the final product of the static, surface culture is a 2-4 centimetres thick cellulose layer situated on the broth surface. Our research is directed to the surface culture because of the larger application potential for membranes of bacterial cellulose, produced in this way.

After the growth of the cellulose layer thickness has ceased, the material can be harvested and cut into slices of the desired thickness. The hydrophilic and mechanical properties of such cellulose membranes provides a wide field of potential applications for this product. As an example we established recently the application of special impregnated cellulose membranes as a high-quality cosmetic product [7] and as a scaffold for animal cell cultures [28]. But there is a major hurdle for the successful large-scale commercialisation of this surface technology for the production of BC. This arises from the low yield of the process and the limited maximum thickness of the cellulose layer.

The low productivity of the surface culture process was described by many other authors in the past. Because of this, a major objective has been the improvement of the product yield. Recently, work elsewhere has been focused on the enhancement of the microbial conversion of the carbon source into bacterial cellulose. Typical examples include:

- Large scale screening of naturally sources for cellulose producing strains and isolation of high rate production strains [9]
- In situ pH control via an optimised fermentation medium design [10]
- Variation of carbon sources (mannitol [11], fructose [12], sucrose [13], arabitol [14], glycerol [15])
- Promotion of cell growth and BC production by adding of supplements or additional substrates to the medium (lactate [16], ethanol [17], chitosan [18], endoglucanase [19])

- Production of *Acetobacter* mutants (deficient in (keto)gluconate synthesis [10], resistant to sulfaguanidine [20])

Fig.1 shows a typical culture tray at the end of cultivation and a cellulose membrane after the downstream processes of slicing and wasing.

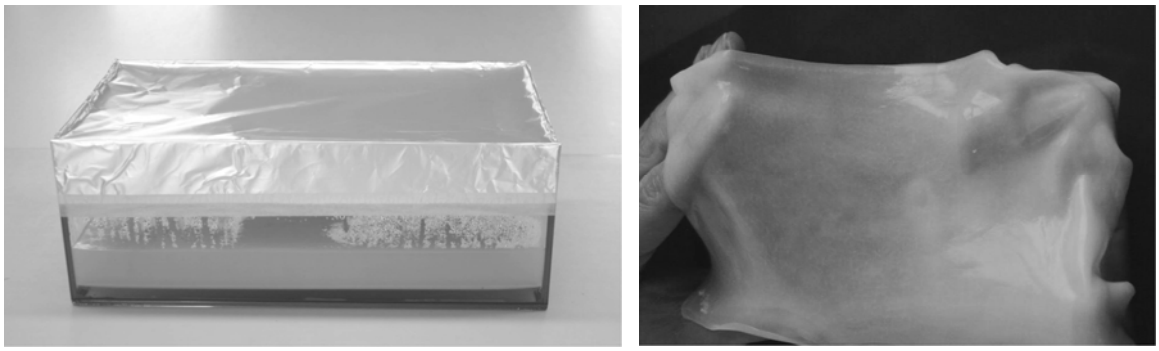

Figure 1. *Culture tray after 30 days of cultivation (maximum cellulose thickness) and cellulose membrane*

The primary goal of this research

This study is dealing with the development of an improved fermentation process for the production of bacterial cellulose in surface culture. The main conclusion that can be deduced from the previous work is that factors other than the microbial rate of production have a major influence on the process. The consequence of this observation is that improvements will be less successful unless other (e.g. transport) processes are included in the investigation. To optimise the production of bacterial cellulose, it is necessary to characterise the microbial growth, product formation and substrate utilisation by the microorganisms. In addition, it is imperative to identify how these processes are affected by external chemical and physical conditions. Chapters 2, 3 and 4 of this study describe the first steps in the optimisation of the biotechnological production of bacterial cellulose in surface cultures including the setting and evaluation of the targets for process optimisation by identification of the factors which dominate the process. The aim of the research is to investigate the influences of:

- Bioreaction (indicated by product formation, bacterial growth and substrate consumption)
- Transport of the carbon source
- Oxygen transport and
- Removal of cellulose fibres

as symbolized in Fig.2.

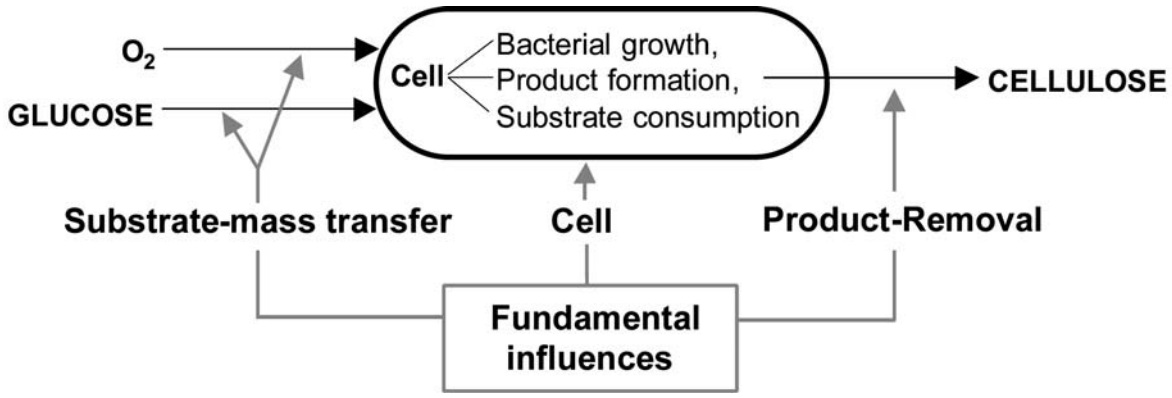

Figure 2. *Fundamental influences on the production of BC in a surface culture*

Up to now, such a complete and complex analysis of the overall system (especially substrate mass transfer and product removal influences) has not been published yet. The usual practice has been to study just one (or two) influences on the process.

2.3 Materials and Methods

This work based on the natural surface culture described by Schramm and Hestrin in 1954 [3] without any supplements to the media, no regulation or buffering of the pH and no genetically modification of the producing strain.

Microorganism

In all experiments, the wild type strain *Gluconacetobacter xylinus* AX5 from the stock collection of the Research Centre for Medical Technology and Biotechnology (Geranienweg 7, D-99947 Bad Langensalza, Germany, phone:+49-3603-833-145, fax:+49-3603-833-150, e-mail: forschungszentrum@fzmb.de) was used [7].

Experimental fermentation and media

The classical Schramm/Hestrin (SH) media with the following composition per liter was used: 20g Glucose; 5 g BactoYeast Extract; 5 g BactoPeptone; 6,8 g Na_2HPO_4*$12H_2O$; 1,115 g Citric Acid. A 400 ml (7,7 cm diameter) beaker was used with 200 ml SH-Medium plus 500 µl of bacterial suspension (turbidity: McFarland 3-4, from the exponential growth phase that equals $1\text{-}4 \cdot 10^7$ cfu/ml). The beakers were incubated at 30°C. The lids of the beakers stopped the entry of contaminating organisms but allowed oxygen to enter the beaker. All media were autoclaved for 20 min at 121°C. The glucose solution was autoclaved separately and added aseptically after the media had been cooled to below 50°C.

Concentration of Glucose

The concentration of glucose in the substrate solution was determined enzymatically by using a test kit for D-Glucose (Boehringer Mannheim, Germany) and a UV/VIS Spectrometer Lambda 12 (Perkin Elmer, USA) at 340 nm.

Diffusion coefficient for Glucose in wet Cellulose

To independently determine the effective diffusion coefficient, a diaphragm cell was used. In this cell, a diffusional glucose flux was set up through a 1mm thick, washed wet cellulose membrane separating two well mixed liquid-filled compartments. The initial glucose concentration was 20g/l in one compartment and 0g/l in the other. The change of the concentrations as a function of time in both compartments were followed until the steady state was reached. The calculation of the diffusion coefficient was done using Fick's law.

Oxygen Profile of the cellulose layer

The oxygen concentration inside a cellulose layer after 20 days of incubation was determined at 25°C using O_2 – sensitive optical glass sensors (microsensor, Presens, Germany) connected to a fibre optic oxygen meter (Microx TX, Presens). The tip of the microsensor has an diameter of 25 µm. The microsensor was observed for correct positioning on the cellulose surface by a microscope (Zeiss, Germany), and driven into the layer by a micromanipulator at 100 µm intervals. The microsensor were calibrated with ambient air (21% O_2) and N_2 saturated water.

Density of cellulose

The density of wet and dry cellulose was determined by examination of weight (Laboratory balance, Sartorius, Germany) and the volume of water which the sample displaced after dipping it into a reservoir of liquid.

Dry mass and thickness of the cellulose layer

For examination of the dry mass, formed cellulose layer was lifted out of the fermenter, and cut into small cubes (approx. 0,5 cm edges). The cubes were washed twice and dried in an Electronic Moisture Analyser MA 30 (Sartorius, Germany). The layer thickness was measured with a ruler.

Growing of immobilised organisms

To release the immobilised organisms from the cellulose matrix, the layer was cut into thin slices of approx. 0,5 mm thickness with a vertical slicer. The strips were treated with ten times of their mass of a physiological sodium chloride solution and agitated strongly for 30 minutes. During this time, the immobilized bacteria were completely extracted (as proven by long-term extraction overnight, which did not gave a higher number of released cells). The numbers of immobilised organisms were determined after they had been released from the cellulose matrix by the use of a counting chamber and a Light Microscope magnified of 600 times (Hund, Deutschland).

In comparison with a submerged culture (where samples can be repeatedly taken under sterile conditions), in a cellulose surface culture, the sampling for production rates etc. will destroy the cellulose layer and finish the experiment. Similarly for the determination of the number of immobilized microorganisms, again the cellulose layer has to be destroyed. The only way to measure the progress of the overall growth is to have many identical experiments carried out in parallel. For example, ten experiments could be started, the first sampled (and destroyed) after two days, then after four days and so on up to twenty days. Hence, one growth culture (or similar) requires many experiments; each data point requires its own experiment. Such data will naturally have more scatter than from a similar, single production run in a submerged fermenter. On this basis even more replicate experiments have

to be realized to reduce this source of error (e.g. see Fig.3 where 32 independent experiments gave just one dry mass – time profile).

Mathematical methods

The curve fitting program TableCurve^{TM}2D was used to fit kinetic models to the experimental data. The set of ordinary differential equations describing the carbon source transport were solved by using the Euler method, and the confirmation of the Euler method was furnished by the MathWorks Matlab – Simulink package.

2.4 Results and Discussion

Product formation and bacterial growth

The product formation within a static surface culture starts with the formation of island-like cellulose fragments on the broth surface. Later, the fragments close together to form a thin cellulose film. The thickness of this layer increases to values between 2 to 3 cm (total cellulose dry mass per beaker: 1,2 –1,6 g) within 2 to 4 weeks. Experimental data of the increase of the dry mass is represented in Fig.3.

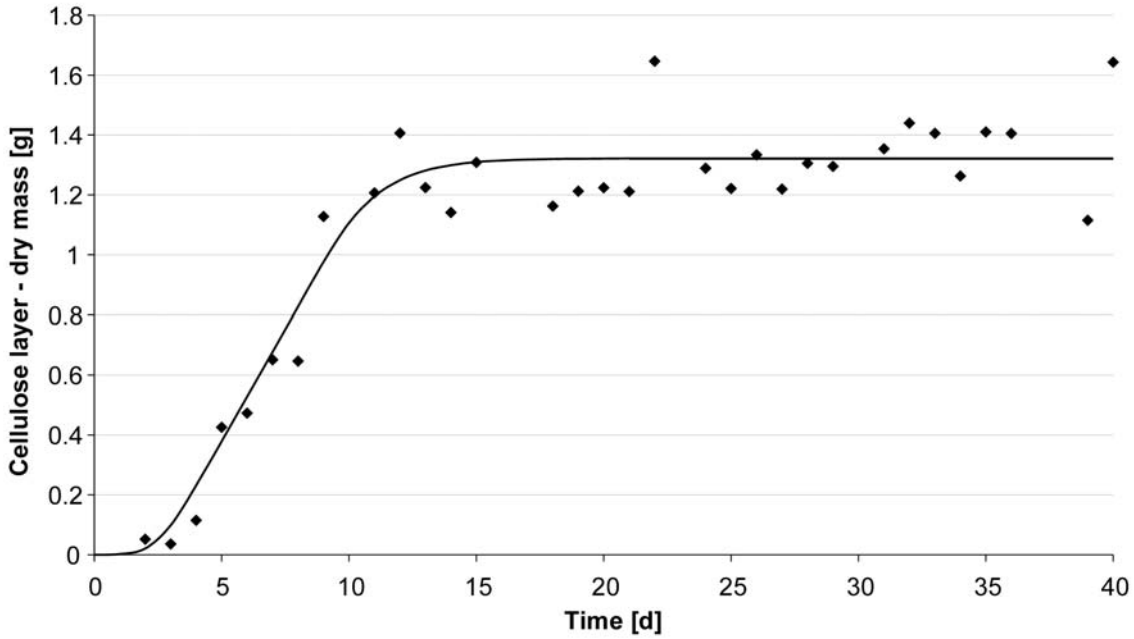

Figure 3. *Layer dry mass during the course of fermentation (from 32 independent measurements)*

The graph of the product formation follows a logistic curve of the form:

$$P(t) = \frac{P_0 e^{\mu_{P,max} t}}{1 - (P_0 / P_{max}) \cdot (1 - e^{\mu_{P,max} t})} \qquad (1)$$

The parameters of the logistic equation are the specific product formation rate $\mu_{P,max}$ [0,55 d^{-1}], the initial amount of product P_0 [0,03 g] (only for calculation) and the maximum attainable product mass

P_{max} [1,3 g]. The shape of P(t) curve has the expected exponential, then linear and then stationary phases.

To date, there have been only a few reports concerning the growth of the bacteria within the cellulose matrix formed in surface culture because of the difficulty in extracting the immobilised microorganisms. Serafica also used this logistic model to describe the increase in the density of the immobilised bacteria [8]. Fiedler et al. found a similar shape for biomass growth [24]. Our examinations fully confirmed the appropriateness of the logistic model (which is very often used for simulation of density-dependent growth of microbial populations, for instance, systems of immobilised bacteria [25]) to describe the growth of immobilised *Gluconacetobacter xylinus* according to equation (2)

$$X(t) = \frac{X_0 e^{\mu_{max} t}}{1 - (X_0 / X_{max}) \cdot (1 - e^{\mu_{max} t})} \qquad (2)$$

where μ_{max} is the specific growth rate, X_0 is the initial amount of biomass and X_{max} the maximum attainable biomass concentration. Fig.4 represents the increase of immobilised bacteria within the cellulose matrix.

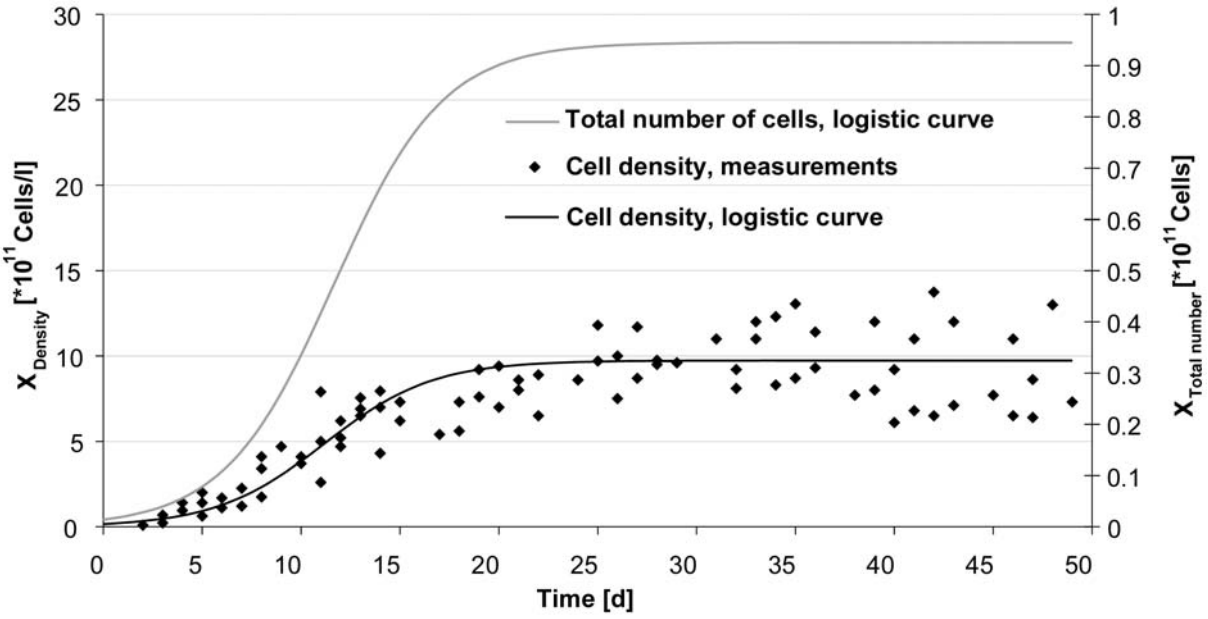

Figure 4. *1. Cell density: ——— Logistic curve: x_0=0,16·10^{11}cells/l, x_{max}=9,7·10^{11}cells/l; μ_{max}= 0,366d^{-1}*

2. Cell number: ——— Logistic curve: x_0=1,37·10^{9} cells; x_{max}=0,95·10^{11}cells; μ_{max}= 0,286d^{-1}

(from more than 80 independent measurements)

In submerged cultures, the amount of the homogenous distributed product is usually quantified as a concentration in g/l. The product of the cellulose surface culture is a layer, which is separate and

distinct from the medium. That is why the unit of total mass (g) is more appropriate here then a concentration. To keep the comparability of the units, the density of cells within the cellulose was also converted by multiplication by the total volume of cellulose layer into a total number of cells (Fig.4, top curve). The graph of total number of immobilized cells again follows a logistic curve.

Oxygen is required for the aerobic growth and the product formation of *Gluconacetobacter xylinus*. Borzani and de Souza [21] and Schramm and Hestrin [3] found by means of tracer experiments (using black hair or particles of cork) that the formation of cellulose occurs only at the upper film/air interface. Since these experiments, it is assumed that the produced cellulose is pushed gradually down, while the new cellulose layers are constantly being built on the top of the mature cellulose.

Our oxygen concentration measurements within a cellulose layer determined a 800–1000 µm thick aerobic upper zone. Further down, anaerobic conditions prevail (see Fig.5).

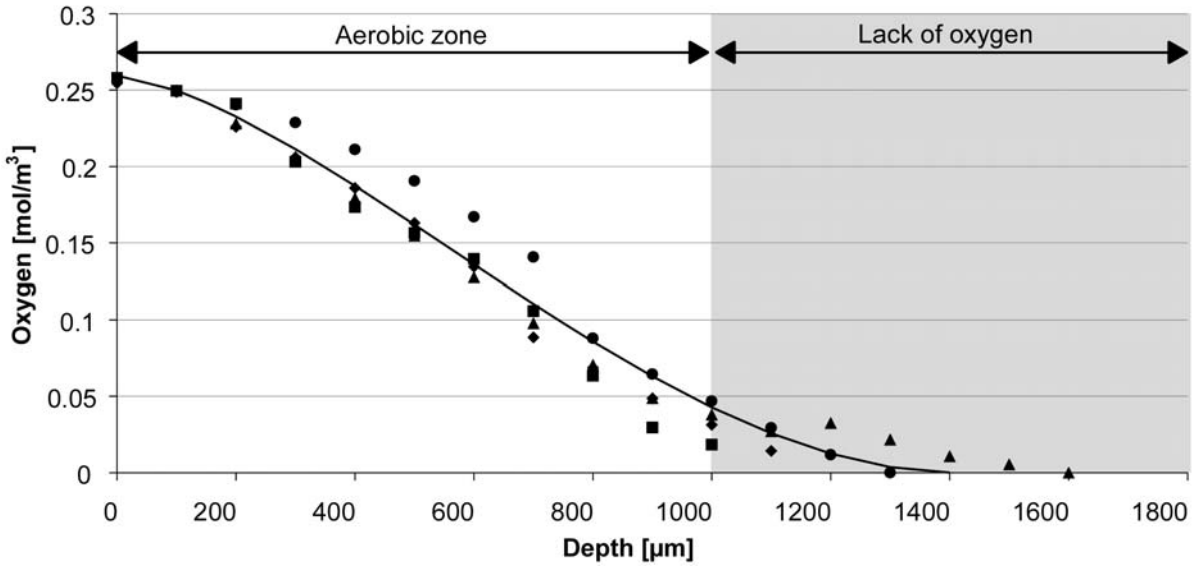

Figure 5. *Oxygen profile within a bacterial cellulose layer (from 4 independent measurements; 0,258mol O_2/m^3=100% O_2 Saturation at 25°C)*

Fig.5 and the older tracer experiments mentioned above, show that the aerobic zone is rather thin, approximately 1 mm in thickness. Only the part of the total number of cells which are immobilized within this aerobic zone, is able to produce cellulose. The bacteria in deeper zones must be in an inactive state and cannot make any more product. Due to the immobilisation of the bacteria between the cellulose fibres and the sinking material during the product formation, the aerobic zone bacteria are gradually drawn into the anaerobic zone of the cellulose layer. This leads us to the following conclusions:

The total cell count is not important. The really significant quantity is the number of cells in the aerobic zone which are producing the cellulose.

The product formation is determined by the growth and capacity for product formation of the bacteria within the aerobic zone.

The number of cellulose producing cells could be expected to be equal to the rate of growth of the total cell number. The producing cells can be estimated from equation (3) which is the differentiated form of the logistic equation (2) (relating to 1 day).

$$\frac{dX}{dt} = \mu_{max} X(t)\left(1 - \frac{X(t)}{X_{max}}\right) \tag{3}$$

Fig.6 shows it graphically, see right hand scale.

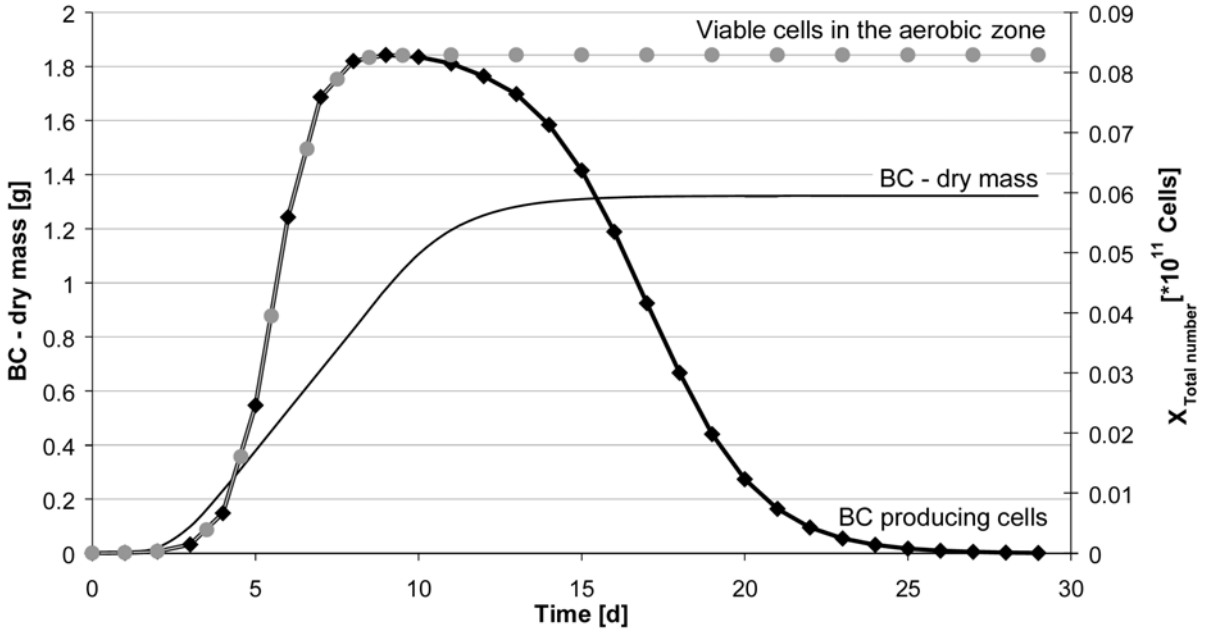

Figure 6. *Course of cellulose producing cells and dry mass of the produced cellulose*

After a brief lag phase, the number of cells which produce BC (black curve) increases exponentially, reaches the maximum value of $8{,}3 \cdot 10^9$ cells and stays constant on the maximum value between days 8 to 10 of incubation. From day 11, the number of BC producing cells seems to decrease due to the cessation of cellulose production.

The calculated reduction in cellulose producing cell numbers seen above results directly from the mathematics of the logistic equation. The stagnation of the product formation may be caused by the reduction of producing cells, but it is not feasible to draw this conclusion without further investigations. In order to discover what actually happens, we need to know if the cells in the aerobic zone are really dying in the later period. An alternative explanation is that there is a constant number of living cells whose capacity to make cellulose is reduced by another influencing factor (Fig.6, grey curve, labelled as viable cells in the aerobic zone). We need to know which theory is true.

Substrate consumption of the bacteria

In this section the glucose consumption of the bacteria within the aerobic cellulose matrix was monitored over a long period of time. Fig.7 shows the dynamics of glucose usage compared to the dry mass of BC and growth of the total cells. These measurements (black squares) show a declining trend, which never flattens out and, as time progresses (t > 25 d) shows a markedly linear trend.

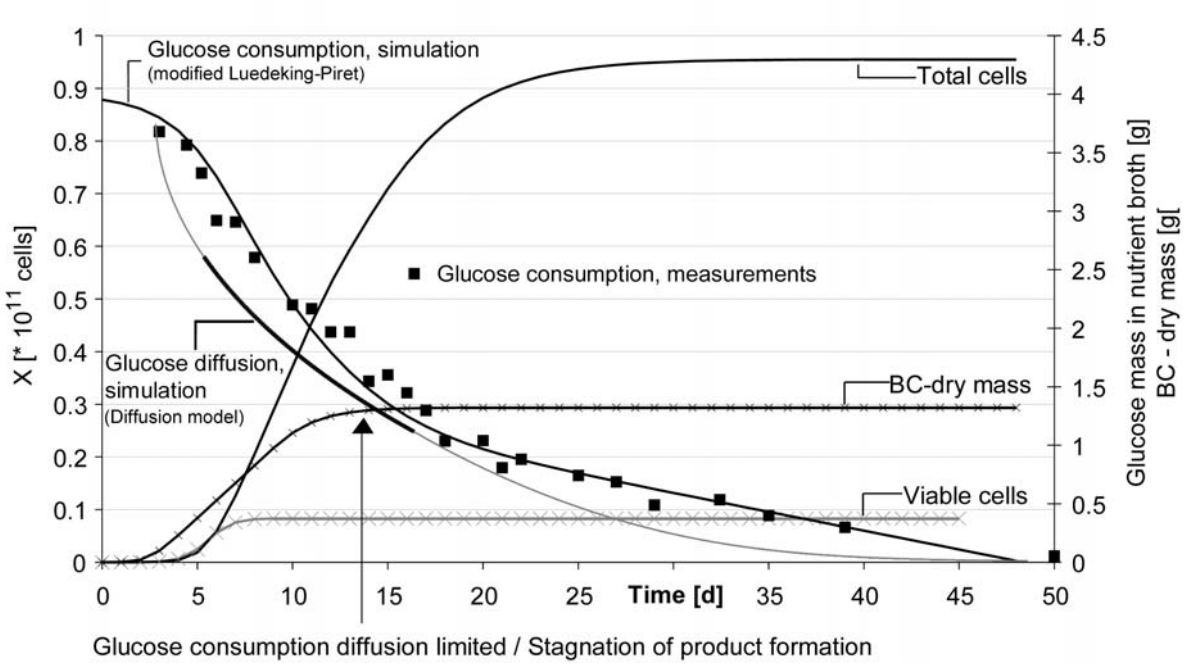

Figure 7. *Time course of glucose consumption (measurements and simulation) compared to cells and BC-dry mass*

The glucose which enters the aerobic zone will consumed by the formation of the cellulose, the growth of the biomass and the maintenance of the viable cells. As Fig.7 shows, the glucose is consumed even when there is no overall cell growth and no increase in the BC dry mass. From day 25 of the incubation, the substrate is being used at a linear rate and this indicates a fixed population of living bacteria in the aerobic zone consuming substrate to maintain themselves.

To illustrate the argument about the constancy of the living cell numbers we can simulate the substrate consumption using the Luedeking-Piret equation. Weiss et. al [27] used a modified form of these equations to describe the course of substrate consumption S(t) in the biotechnological production of the exopolysaccharide xanthan, where the substrate is consumed for growth, product formation and biomass maintenance. An appropriate kinetic form is therefore:

$$S(t) = \underbrace{Y_{\frac{S}{X_{TC}}} \cdot X_{TC}(t)}_{\text{(Growth of total cells, TC)}} + \underbrace{Y_{\frac{S}{P}} \cdot P(t)}_{\text{(Product formation)}} + \underbrace{k_e \cdot \int_0^t X_{VC}(t)dt}_{\text{(Maintenance of viable cells, VC)}} \quad (4)$$

Using equation (4), we can substitute the two logistic approximations for growth of the total number of immobilized bacteria [see Fig.7, total cells and equation (5)] and the product formation [see Fig.7, BC-dry mass and equation (6)] and the integrated form of the logistic growth of the viable cells [see Fig.7, viable cells and equation (7)] as listed below:

$$X(t) = \frac{X_0 e^{\mu_{TC,max} t}}{1-(X_{TC,0}/X_{TC,max})\cdot(1-e^{\mu_{TC,max} t})} \tag{5}$$

$$P(t) = \frac{P_0 e^{\mu_{P,max} t}}{1-(P_0/P_{max})\cdot(1-e^{\mu_{P,max} t})} \tag{6}$$

$$\int_0^t X_{VC}(t)dt = \frac{X_{VC,max}}{\mu_{VC}} \cdot \ln\left(1-\frac{X_{VC,0}}{X_{VC,max}}\cdot(1-e^{\mu_{VC} t})\right) \tag{7}$$

Tab.1 gives the parameters we used for the simulation of substrate consumption.

Parameter		Value and unit	
Total number of immobilized cells			
$X_{TC,0}$	Initial number of total cells	$1{,}37\cdot10^{9}$	cells
$X_{TC,max}$	Final number of total cells	$0{,}94\cdot10^{11}$	cells
$\mu_{TC,max}$	Max. growth rate of total cells	0,36	d^{-1}
$Y_{S/X(TC)}$	Yield coefficient (g substrate/cell)	$1{,}22\cdot10^{-11}$	g/cell
Product formation			
P_0	Initial amount	0,0281	g
P_{max}	Final amount	1,322	g
$\mu_{P,max}$	Max. product formation rate	0,552	d^{-1}
$Y_{S/P}$	Yield coefficient (g substrate/g product)	1,11	---
Growth of viable cells			
$X_{VC,0}$	Initial number of viable cells	$1{,}19\cdot10^{6}$	cells
$X_{VC,max}$	Final number of viable cells	$0{,}08\cdot10^{11}$	cells
$\mu_{VC,max}$	Max. growth rate of viable cells	1,6	d^{-1}
k_e	Maintenance rate [g substrate/(viable cell·d)]	$3{,}86\cdot10^{-12}$	g/cell·d

Table 1. *Parameters for simulation of substrate consumption (using modified Luedeking-Piret model)*

The maintenance coefficient k_e was determined by fitting a linear function to the experimental data of the glucose consumption between 25-40 d of incubation. After 25 days of incubation, the total number of cells and cellulose dry mass have reached their maximum values. That means the glucose will be consumed only for maintaining the viable cells. The yield coefficient $Y_{S/X(TC)}$ was calculated by fitting equation (4) to the experimental data of glucose consumption. The yield coefficient $Y_{S/P}$ was calculated from the condensation polymerisation of glucose into cellulose which is 1 mol glucose [M_{Glc} = 180 g] giving 1 mol of anhydrous-glucose units [M_{Glc} - M_{H2O} = 162 g], the basic units of cellulose [180 g/ 162 g = 1,11].

The result of the simulation is displayed in Fig.7. The modified Luedeking-Piret equation, gives a good fit to the data, again confirming our assumption that the number of living cells (approximately 10% of the total cells) is constant (Fig.6 grey bacteria curve). But it appears that the product forming capacity of the living cells becomes limited.

In the next section we will consider, if insufficient substrate supply of the living cells causes the decrease of product forming capacity. We can study the two main substrates glucose and oxygen.

Oxygen diffusion

The oxygen profile (see Fig.5) determined a 800-1000 µm aerobic upper-zone in which oxygen will be consumed by the aerobic cells. An insufficient diffusional oxygen flux from the air-space above the aerobic zone into the aerobic zone would cause a drop of the dissolved oxygen level below a critical concentration. Under these conditions, the cells would be oxygen limited and the rate of product formation would decrease. In order to check if oxygen supply was a problem, the head space of the beaker above the cellulose was periodically (10 min/h) fed with fresh sterile air (not enriched with oxygen). This made no difference. The conclusion must be that the stagnation of product formation is not a result of oxygen limitation.

Glucose diffusion

We have proved that active cells exists only at the top layer of the cellulose (max. 1mm thickness) because of oxygen limitations in deeper zones (see Fig.5). For the active cells within the aerobic cellulose matrix, nutrients have to diffuse from the substrate solution below the cellulose through the oxygen limited cellulose matrix as displayed in Fig.8. In the opinion of several authors [8, 23] the basic disadvantage of the static tray culture is that the floating cellulose layer becomes a barrier for substrate mass transfer in later stages of incubation. The question we want to answer now is if the glucose mass transfer limitations is indeed caused by the increasing cellulose layer thickness.

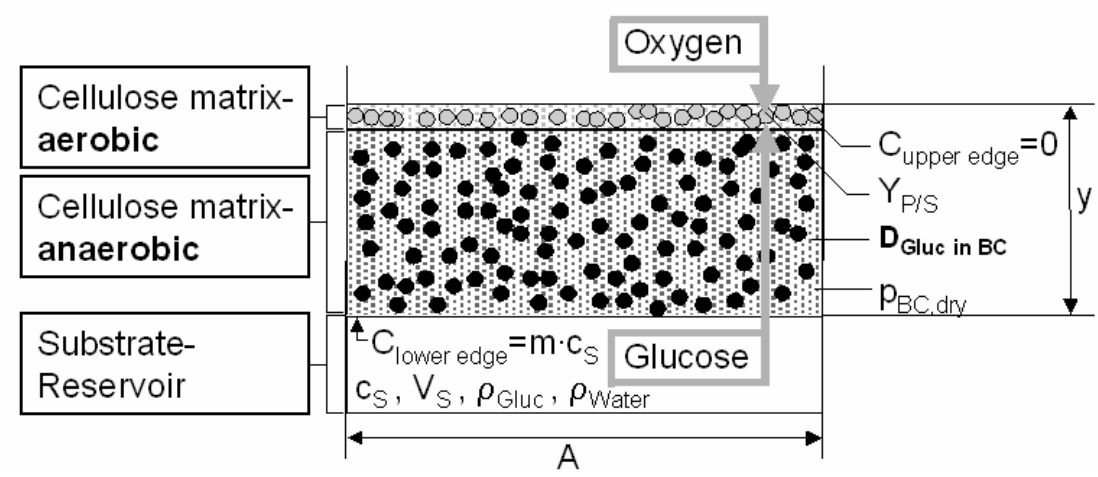

Figure 8. *Cross-section of a static bacterial cellulose culture (grey circles: bacteria within the aerobic zone; black circles: bacteria within the anaerobic zone)*

To answer this question, the manufacture of bacterial cellulose has been modelled by a set of ordinary differential equations representing the glucose, water and cellulose balances. Each has the following form

$$\mathrm{ACC_j} = \mathrm{IN_j} - \mathrm{OUT_j} + \mathrm{GEN_j} \tag{8}$$

where ACC_j is the rate of accumulation of a substance j in a volume element. IN_j and OUT_j are the rates of inflow and outflow of the substance j in the volume and GEN_j is the generation rate of substance j within the volume element [26].

We envisage a horizontal tray of area, A, which initially contains the glucose solution. The cellulose is formed at the upper surface and the thickness, y, increases with time, t. As time progresses, there is an increasing thickness of cellulose through which the glucose must diffuse. We assume that:

The rate determining step is diffusion. This means that the reaction rate for the conversion of glucose into cellulose within the aerobic cellulose matrix is practically instantaneous. We note that the glucose is totally used up at the upper edge , see Fig.8. (However, during the early lag phase growth of the living cells, this may be somewhat in error.)

The glucose within the substrate layer is assumed to be well mixed, because the diffusion coefficient of glucose in water ($D_{gluc\ in\ water} = 6 \cdot 10^{-10}$ m^2/s) is bigger than that for glucose in cellulose ($D_{gluc\ in\ cellulose} = 4{,}05 \cdot 10^{-10}$ m^2/s).

There is no consumption of glucose in the substrate reservoir because of the anaerobic-like conditions (there are no anaerobic bacteria within the substrate reservoir).

Doing a glucose balance on the substrate layer we have:

$$\frac{d\left[V_S(t)\cdot c_S(t)\right]}{dt} = 0 - A\cdot D\cdot\left.\frac{dc}{dy}\right|_{\text{lower edge}} + 0 \qquad (9)$$

and

$$\frac{d\left[V_S(t)\cdot c_S(t)\right]}{dt} = V_S\cdot\frac{dc_S}{dt} + c_S\cdot\frac{dV_S}{dt} \qquad (9a)$$

where D is glucose diffusivity within the cellulose matrix, c is the glucose concentration within the cellulose and c_S is its concentration in the substrate layer, whose total volume is V_S. We now assume a quasi steady state glucose profile in the cellulose matrix, leading to a linear concentration variation through its depth (background Fick´s first law of diffusion). At the top surface, we assume a rapid bioreaction which consumes the glucose ($c_{\text{upper edge}} = 0$).

That gives

$$\left.\frac{dc}{dy}\right|_{\text{lower edge}} = \frac{c_{\text{lower edge}} - 0}{y - 0} = \frac{m\cdot c_S}{y} \qquad (9b)$$

because we define $c_{\text{lower edge}}=m\cdot c_s$.

$$\frac{dc_S}{dt} = \left[\frac{-A\cdot D\cdot m\cdot c_S}{y} - c_S\cdot\left(\frac{dV_S}{dt}\right)\right]\cdot\frac{1}{V_S} \qquad (9c)$$

A glucose balance on the cellulose layer is given by

$$0 = \frac{A\cdot D\cdot m\cdot c_S}{y} - 0 - A\cdot\left(\frac{dy}{dt}\right)\cdot\rho_{BC}\cdot p_{BC,dry}\cdot\frac{100}{Y_{P/S}} \qquad (10)$$

where ρ_{BC} is the overall density of the cellulose layer. $p_{BC,dry}$ is the fraction of the layer which is cellulose and $Y_{P/S}$ is a yield coefficient describing the part of the diffusing glucose which is converted into cellulose. After rearrangement we obtain:

$$\frac{dy}{dt} = \frac{D\cdot m\cdot c_S\cdot Y_{P/S}}{y\cdot\rho_{BC}\cdot p_{BC,dry}\cdot 100} \qquad (10a)$$

Finally, the volume of the substrate layer changes thus

$$\frac{dV_S}{dt} = \frac{-A \cdot D \cdot m \cdot c_S}{y \cdot \rho_{Glucose}} - \frac{A \cdot D \cdot m \cdot c_S}{y \cdot \rho_{Water}} \cdot \frac{(1 - p_{BC,dry})}{p_{BC,dry}} \tag{11}$$

where $\rho_{Glucose}$ and ρ_{Water} are the densities of pure glucose and pure water, respectively.

Equations (9c), (10a) and (11) constitute a set of ordinary differential equations which can be conveniently solved by a simple Euler approach from the known initial conditions for y, V_S and c_s. Table 2 gives the parameter values we used in the model. D, $Y_{P/S}$, $p_{BC,dry}$ and ρ_{BC} were estimated independently in our laboratory.

Parameter		Value and unit	
A	Surface	0,004657	m^2
$C_{S,0}$	Initial substrate reservoir Glucose concentration	20	kg/m^3
D	Diffusion coefficient of glucose in cellulose	4,05·10-10	m^2/s
m	Equilibrium constant	1	---
$Y_{P/S}$	Yield coefficient (glucose converted into cellulose)	45	%
$p_{BC,dry}$	Dry cellulose fraction	0,01	---
$V_{S,0}$	Initial substrate reservoir volume	0,0002	m^3
ρ_{BC}	Density of cellulose	1030	kg/m^3
ρ_{Water}	Density of water	1000	kg/m^3
$\rho_{Glucose}$	Density of glucose	1550	kg/m^3

Table 2. *Parameter values for simulation of glucose diffusion*

Fig.7 gives the result of the simulation. As we have discussed, the glucose diffuses through the growing cellulose layer. The prediction from the diffusion model is shown as a full grey/black line. The model slightly over-estimates the consumption of glucose at the early stage of incubation (as predicted earlier, until X is close to X_0). The prediction of the Luedeking-Piret Simulation of the substrate consumption is also shown. Interestingly the two sets of predictions are close at around 15 days, when cellulose dry-mass curve flattens out. This suggests that the effect of glucose mass transfer diffusion is limiting the formation of the cellulose. (The glucose which continues to be consumed after the stagnation of product formation is used for cell maintenance.)

One way to improve the situation would be to increase the diffusional driving force. On the tenth day of fermentation, the originally medium (20 g Glucose/l) was replaced carefully by a 40 g/l glucose

solution. This date was chosen because it occurs before the mass transfer limitation affects the cellulose production.

Fig.9 shows the result of this intervention. The lower line is the originally (20 g/l) condition. The middle curve shows the measured effects of the change in substrate concentration on day ten, whereas the top curve gives the prediction from the diffusional model.

As can be seen, the theoretical model somewhat over-estimates the growth of the layer and is a shallow curve (almost linear). The two graphs of experimental values show that there is a benefit to increasing the reservoir glucose concentration because the final thickness is increased and the onset of the stagnation is delayed. But the measured 20 to 40 g/l line does become horizontal, in contrast to the model's prediction. Hence, we can conclude that there is yet another significant influence on the fermentation to be determined, in addition to the real effect of the diffusional limitation.

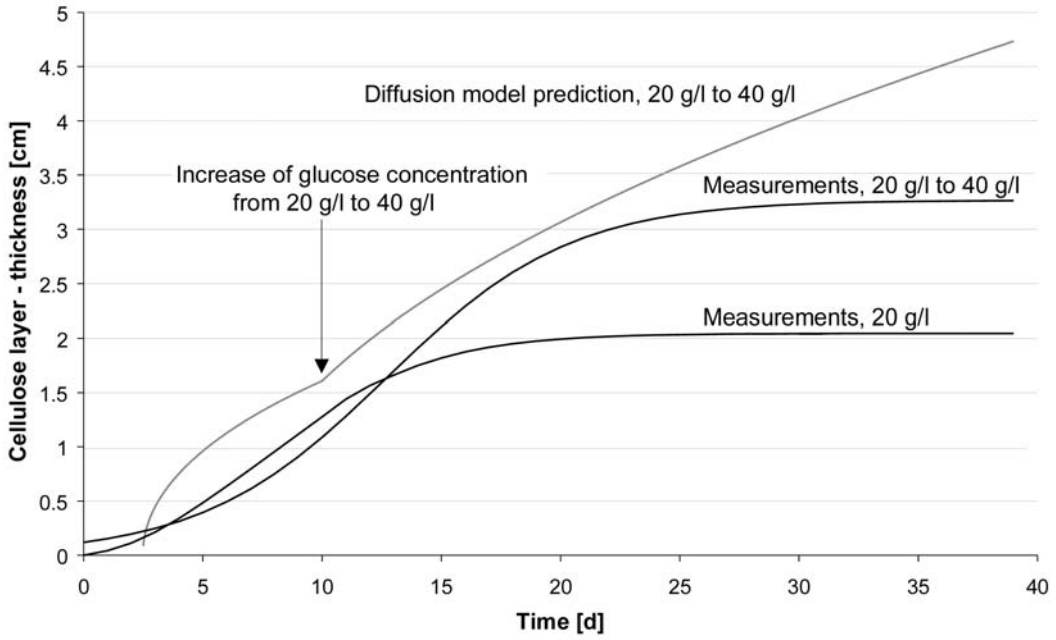

Figure 9. *Comparison of experimental data $c_{S,0}$=20g/l Glucose [lower curve]; $c_{S,0}$=20g/l Glucose →$c_{S,0}$=40g/l Glucose at 10th day [middle curve] and prediction using the Diffusion model $c_{S,0}$=20g/l Glucose →$c_{S,0}$=40g/l at 10th day [top curve]*

2.5 Conclusion

The paper has studied the microbial production of cellulose in surface culture fermentation. The main feature is the performance of the immobilized bacteria in the cellulose layer. The growth of both product and bacteria, plus the consumption and supply of substrate have been measured and modelled.

The results of O_2 profile measurements showed that the strictly aerobic condition for the growth of the bacteria only exist in a thin, upper surface layer of the cellulose. The evaluation of the cell number and of glucose usage rates has shown that, at most, 10% of the total bacterial cells are active and this number remains constant.

However, the cellulose growth rate ceases after about 15 days. The theoretical model based on diffusional resistance together with the Luedeking-Piret simulation of substrate consumption at around 15 days indicates the importance of mass transfer limitations at this time. The experiment involving changing the concentration driving force showed that, in addition to the mass transfer limitations, there is another factor which influences the system, which has yet to be determined.

In the next part of this study, we shall consider the effects of the removal of the product (see Fig.2) to explain the discrepancies described above.

Acknowledgements
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3 DYNAMIC MODELLING OF BACTERIAL CELLULOSE FORMATION

3.1 Abstract

The interest in cellulose produced by bacteria from surface cultures has increased steadily in recent years because of its potential for use in medicine and cosmetics. Unfortunately, the low yield of this production process has limited the commercial usefulness of bacterial cellulose. The aim of this chapter is to show the effect of substrate mass transfer on the growth of the bacteria and on their physiological potential for product formation by means of a dynamic mathematical model.

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3.2 Introduction

In recent years there has been an increasing interest in biotechnologically produced polymers. Besides products like polyhydroxybutyrate (PHB) and 1,3-propanediol (PDO), bacterial cellulose in the form of thin membranes is a most promising material. The interest results from its hydrophilic and mechanical properties (see the network structure in Fig.1, right) of such cellulose membranes, which provide a wide field of potential applications for this product. As an example, we recently established the application of specially impregnated cellulose membranes as a high-quality cosmetic product [1] and as a scaffold for animal cell cultures [2].

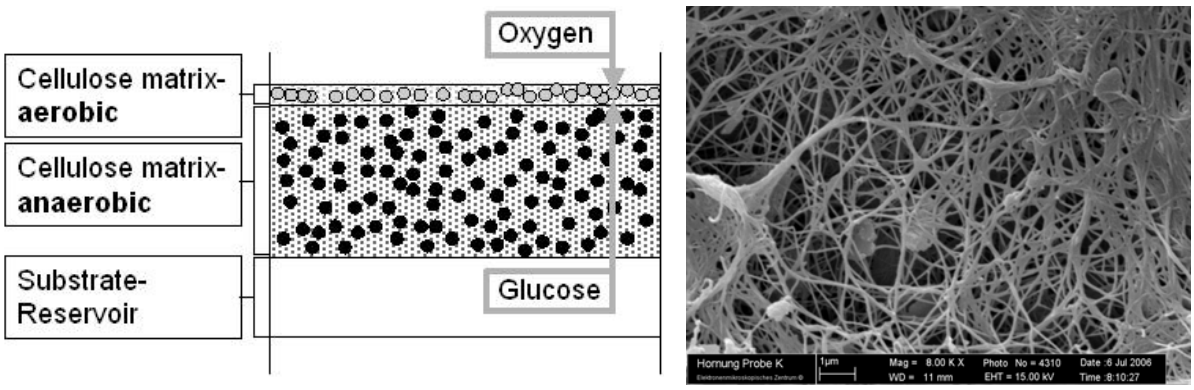

Figure 1. *Cross-section of a static bacterial cellulose culture (left); Scanning Electron Microscopy of BC-fibres (right)*

Manufacturing stable bacterial cellulose membranes is only possible when product formation takes place in surface cultures (Fig.1, left). Bacterial cellulose formation in surface cultures is a complex discontinuous process that is usually performed in rectangular culture boxes. These boxes are filled with a nutritive medium under sterile conditions, see Hestrin and Schramm [3] and inoculated using a suspension of *Gluconacetobacter xylinus*. In order to protect the cells from infections, the culture boxes must be sealed. The bacteria are cultured without agitation at a temperature of 30°C. Oxygen supply is made via the headspace of the boxes. After a delay time of three days, cellulose formation becomes visible. In the beginning, island-shaped fragments form a coherent cellulose layer on the liquid surface. The layer thickness increases and reaches 3–4 centimetres within 3–4 weeks. After harvesting, this cellulose layer can finely be sliced into membranes of the desired thickness.

The traditional surface procedure is inefficient with respect to mass-production, particularly the limited product yields and the labour-intensive preparation steps reduce the efficiency. For this reason, a detailed analysis of this bioprocess was performed in order to improve the production process.

Often, such optimisation procedures are carried out empirically by trial and error, which is a lengthy and cumbersome process due to the high complexity of bioprocesses. The engineering alternative is describing the bioprocess by means of mathematical models and using the models for numerical optimization. The procedure alone of building up a mathematical model by systematic investigation of sub-steps of the process leads to a deeper understanding of the entire process. Further, simulation studies can help analyzing the complex interrelationships within a bioprocess. Generally, the model-based optimization is an engineering approach where the experimental effort can be minimized

significantly compared to the empirical approach. This leads to an accelerated bioprocess development.

The surface culture for the production of bacterial cellulose is a system, in which the producing cells are immobilized inside a matrix formed by the cellulose fibres. Cell growth and product formation are significantly influenced by mass-transport phenomena. In [4] it was shown that the bacteria are homogeneously distributed in the growing cellulose layer. However, product formation is only possible by cells, that are located at the aerobic interface (compare chapter 2 [4], Fig.5 and 8). In the first instance, the substrate which was dissolved in the reservoir below the cellulose layer must diffuse through the entire cellulose layer, in order to reach the active bacteria at the surface of the cellulose layer (aerobic zone). By means of a quasi-stationary diffusion model first published in [4], it was shown that the diffusion controlled substrate supply of the product forming cells has a limiting influence on the product formation rate. It is presently not possible to initiate a basic optimization of the process by targeted improvements of the diffusion requirements. The obvious discrepancy between the model simulation and the measurement (compare chapter 2 [4], Fig.9) led to the following conclusion: the central assumption that the substrate diffusion is the only limiting and therefore rate determining process, is not appropriate. Rather it had to be assumed that the formation of bacterial cellulose in surface cultures is affected also by other factors.

The correctness of this conclusion will be proven here by a second, independent mathematical model. In the above mentioned quasi-stationary diffusion model, only substrate transport phenomena are considered. However, it could also be possible that the growth of the bacteria and their physiological potential for product formation limit the process. Hence, from this point of view, we firstly developed the detailed dynamic model described here. In this model both of these processes, that is the substrate influence and the cell number including the product formation (because this is strongly growth associated), are considered.

3.3 Material and Methods

This work is based on the natural surface culture described by Schramm and Hestrin in 1954 [5] without any supplements to the media, no regulation or buffering of the pH and no genetically modification of the producing strain.

Microorganism

In all experiments, the strain *Gluconacetobacter xylinus* DSM 13368 selected from the wild-type strain AX5 was used. The inoculum was prepared from the strain DSM 13368 of the stock collection of the Research Centre for Medical Technology and Biotechnology was used [1].

Experimental fermentation and media

The classical Schramm/Hestrin (SH) medium with the following composition per liter was used: 20 g Glucose; 5 g BactoYeast Extract; 5 g BactoPeptone; 6,8 g Na2HPO4*12H2O; 1,115 g Citric Acid. A 400 ml (7,7 cm diameter) beaker was used with 200 ml SH-Medium plus 500 µl of bacterial suspension (turbidity: McFarland 3-4, from the exponential growth phase that equals $1\text{-}4 \cdot 10^7$ cfu/ml). For some experiments, the amount of glucose and the volume of nutrient broth were varied. The

beakers were incubated at 30°C. The lids of the beakers stopped the entry of contaminating organisms but allowed oxygen to enter the beaker. All media were autoclaved for 20 min at 121°C. The glucose solution was autoclaved separately and added aseptically after the media had been cooled to below 50°C.

Concentration of Glucose
The concentration of glucose in the substrate solution was determined enzymatically by using a test kit for D-Glucose (Boehringer Mannheim, Germany) and a UV/VIS Spectrometer Lambda 12 (Perkin Elmer, USA) at 340 nm.

Diffusion coefficient for Glucose in wet Cellulose
To independently determine the effective diffusion coefficient, a diaphragm cell was used. In this cell, a diffusive glucose flux was set up through a 1 mm thick, washed wet cellulose membrane separating two well mixed liquid-filled compartments. The initial glucose concentration was 20 g/l in one compartment and 0 g/l in the other. The change of the concentrations as a function of time in both compartments was followed until the steady state was reached. The calculation of the diffusion coefficient was done using Fick's law.

Growing of immobilised organisms
To release the immobilised organisms from the cellulose matrix, the layer was cut into thin slices of approx. 0,5 mm thickness with a vertical slicer. The strips were treated with ten times of their mass of a physiological sodium chloride solution and agitated strongly for 30 minutes. During this time, the immobilized bacteria were completely removed (as proven by long-term extraction overnight, which did not give a higher number of released cells). The numbers of immobilised organisms were determined after they had been released from the cellulose matrix by the use of a counting chamber and a Light Microscope magnified of 600 times (Hund, Deutschland).

In contrast to the analysis of submerged cultures (where samples can be repeatedly taken under sterile conditions), in a cellulose surface culture, the sampling for cell numbers etc. will destroy the cellulose layer and finish the experiment. The only way to measure the progress of the overall growth is to have many identical experiments carried out in parallel. For example, ten experiments could be started, the first sampled (and destroyed) after two days, the next after four days and so on. Hence, one growth culture (or similar) requires many experiments; each data point requires its own experiment. Such data will naturally have more scatter than from a similar, single production run in a submerged fermenter. On this basis, even more replicate experiments have to be realized to reduce this source of error (e.g. see Fig.2E where more than 60 independent experiments gave just one cell number – time profile. But this new applied extensive and complicated system allows a safe application of mathematical methods for a better understanding of the emerged cellulose-forming process.

Mathematical methods
The dynamic model was implemented in Matlab and solved using the integration routine ode15s. This routine solves stiff differential equations and differential algebraic equations and uses variable order differentiation formulae.

3.4 Results

Analyses and development of a mathematical model

As a basis for a better understanding and regulation of the complex system of the surface culture for the production of bacterial cellulose a detailed analysis and mathematical interpretation of the results plays a central role. Some information about the process was obtained by means of a quasi-stationary diffusion model [4]. Detailed data about the dynamics of the process and their representation in form of a clear, understandable and realistic model are not yet known.

The basic simplifying assumption of the model is that the entire process is divided into three compartments according Fig.1, left.

Only the upper layer or aerobic zone contains enough oxygen to support biomass growth. Thus, we observe substrate consumption and product formation only in this section. This layer is assumed to be of constant size.

The second compartment contains inactive cells immobilized within the cellulose fibres. This section is growing with time. The cells in this layer do not grow because oxygen is lacking. The compartment is merely considered as a transport resistance to the glucose transport from the third into the first compartment. Molecular diffusion is assumed as the only transport mechanism. Hence, the concentration difference between the upper aerobic zone and the lower compartments is the driving force. During the process, the second layer is continuously enlarged by the cells and the cellulose fibres they produce.

The third compartment is the substrate reservoir. Its substrate concentration is depleted by means of the glucose transport into the aerobic zone.

All variables used for the development of this model are summarized in Tab.1. The basic process is outlined in Fig.1 and described by the equations (1)–(18).

The cells inside of the aerobic cellulose matrix grow based on equation (1).

$$\frac{dn_{X,a}}{dt} = \mu \cdot n_{X,a} \tag{1}$$

Including consideration of the maintenance metabolism of the cells, the following equation (2) for endogenous metabolism is a useful extension of equation (1) [6].

$$\frac{dn_{X,a}}{dt} = \left(\mu - k_e \cdot Y_{XS}\right) \cdot n_{X,a} \tag{2}$$

It is assumed that the aerobic zone grows up to a final strength in which the cells can sufficiently be supplied with oxygen. This leads to a maximal cell density of $c_{X,a,max}$ and the culture reaches a steady state: With new cells formed in better aerated parts of the layer, already existing cells at the lower edge of compartment one become insufficiently aerated and hence, become part of compartment two. In this way, after establishing the steady state of the aerobic zone, its size and the cell density remains constant while the size of the second compartment, the cellulose layer with immobilized non-growing cells is steadily becoming larger. This knowledge was substantiated by our modified sampling and measuring technology giving newer insights into the process. This behaviour can be described by the logistical growth model, which extends equation (2).

$$\frac{dn_{X,a}}{dt} = \left(\mu - k_e \cdot Y_{XS}\right) \cdot \left(1 - \frac{c_{X,a}}{c_{X,a,max}}\right) \cdot n_{X,a} \tag{3}$$

For model simulation equation (3) was used.

Due to the limitation of oxygen in the deeper layers of cellulose (compare chapter 2, Fig.5), the cell numbers only increase in the aerobic zone. This implies that the increase of the total number of cells may be described with the help of the equation (4).

$$\frac{dn_X}{dt} = \dot{n}_{X,a} = \left(\mu - k_e \cdot Y_{XS}\right) \cdot n_{X,a} \tag{4}$$

For the amount of glucose in the reservoir the balance equation (5) is valid, where J_G describes the transport of glucose by diffusion.

$$\frac{dm_G}{dt} = -J_G \tag{5}$$

The amount of glucose in the aerobic cellulose matrix is reduced by the consumption of the cells immobilized in the aerobic zone. This consumption is balanced by inflow from glucose diffusing out of the reservoir.

$$\frac{dm_{G,a}}{dt} = J_G - q_G \cdot n_{X,a} \tag{6}$$

The diffusion through the cellulose layer can be described after Fick`s law of diffusion by the following approach:

$$J_G = D \cdot A \cdot \frac{c_G - c_{G,a}}{y} \tag{7}$$

Glucose is consumed for cells growth and product formation, as described in equation (8):

$$q_G = \frac{\mu}{Y_{XS}} + \frac{q_{BC}}{Y_{PS}} \tag{8}$$

Cellulose is produced proportionally to the amount of cells in the aerobic zone:

$$\frac{dm_{BC}}{dt} = q_{BC} \cdot n_{X,a} \tag{9}$$

Note that the cells in the part below the aerobic zone are not able to use glucose for growth or product formation because of the strong limitation of oxygen.

In order to describe the specific growth rate, the Monod equation (10) is assumed:

$$\mu = \mu_{\max} \cdot \frac{c_{G,a}}{c_{G,a} + K_G} \tag{10}$$

The specific product formation rate is growth dependent and can be described by the following equation (11):

$$q_{BC} = Y_{PX} \cdot \frac{c_{G,a}}{c_{G,a} + K_P} \cdot \mu \tag{11}$$

The volume of the cellulose layer arises from (12) as

$$V_{BC} = \frac{m_{BC}}{\rho \cdot f_{BC}} \tag{12}$$

and the layer thickness is best described as

$$y = \frac{V_{BC}}{A} \tag{13}$$

The concentrations can be calculated by

$$c_{G,a} = \frac{m_{G,a}}{V_a} \quad ; \quad c_G = \frac{m_G}{V}$$

$$c_{BC} = \frac{m_{BC}}{V} \quad ; \quad c_X = \frac{n_X}{V_{BC}} \quad ; \quad c_{X,a} = \frac{n_{X,a}}{V_a} \tag{14-18}$$

Symbol	Notation	Value / Unit
A	area of cellulose layer	0.004657 m^2
D	diffusion coefficient of glucose in cellulose layer	$4.5 \cdot 10^{-10}$ m^2/s
c_{BC}	cellulose concentration	g/l
c_G	glucose concentration in reservoir	g/l
$c_{G,a}$	glucose concentration in the aerobic zone	g/l
c_X	total density of cells	10^9 cells/l
$c_{X,a}$	cell density in aerobic zone	10^9 cells/l
$c_{X,a,max}$	maximum cell density in the aerobic zone	$1163 \cdot 10^9$ cells/l
f_{BC}	dry cellulose fraction	0.01
y	cellulose layer thickness	m
J_G	flux of glucose through the cellulose layer	g/d
K_G	saturation constant of glucose	0.04 g/l

K_P	saturation constant of cellulose	0.01 g/l
k_e	maintenance rate	0.003 g/d·10^9 cells
m_{BC}	mass of cellulose	g
m_G	mass of glucose amount in reservoir	g
$m_{G,a}$	glucose amount in the aerobic zone	g
n_X	fotal number of cells	10^9 cells
$n_{X,a}$	cell number in aerobic zone	10^9 cells
q_{BC}	specific cellulose formation rate	g/d·10^9 cells
q_G	specific glucose consumption rate	g/10^9 cells
V	working volume	l
V_a	aerobic reaction volume at the surface	0.005 l
V_{BC}	volume of cellulose layer	m^3
Y_{XS}	yield coefficient of biomass/glucose	45·10^9 cells/g
Y_{PS}	yield coefficient of cellulose/glucose	0.9·g/g
Y_{PX}	yield coefficient of cellulose/biomass	0.01125g/10^9 cells
μ	specific growth rate	1/d
$\mu_{\max}$	maximum specific growth rate	1.6 1/d
ρ	density of cellulose layer	1030 kg/m3

***Table 1**. Model variables*

Adaptation of the model parameters to the experimental data and evaluation of the model

The model parameters were adapted to the experimental data. The results of the comparison of measured data with the simulation of this dynamic model are reflected graphically in Figure 2A-E.

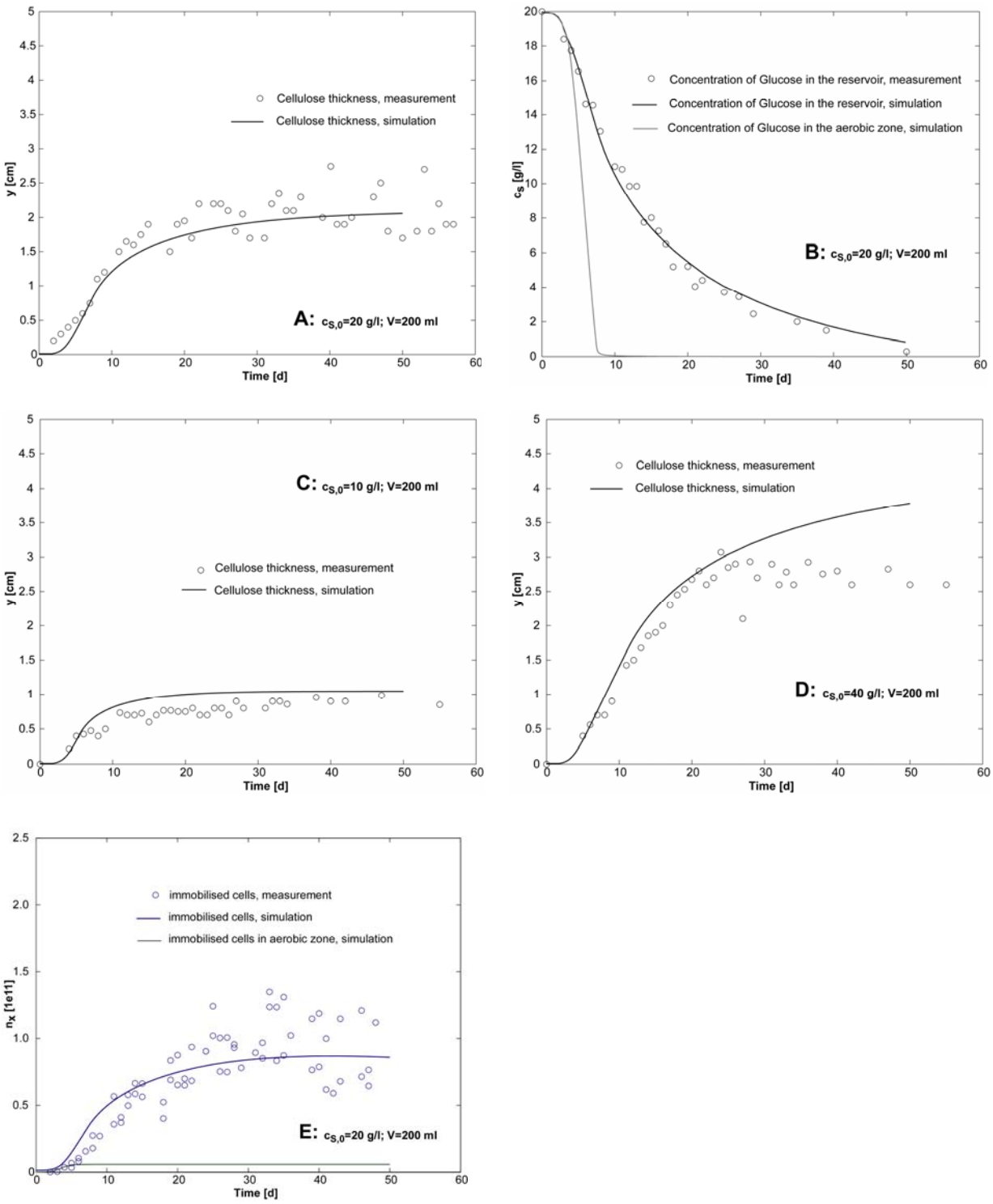

Figure 2. *Comparison of experimental data and simulation at different starting conditions*

The results in Fig.2A demonstrate that the development of the bacterial cellulose layer thickness and the number of immobilised cells (Fig.2E) for the case $c_{S,0}$ = 20 g/l (the usual starting concentration) can be described very appropriately by the dynamic model. The simulated predictions also match the measurement of glucose concentration in the reservoir (Fig.2B) in an excellent manner. Additionally, Fig.2B indicates the simulated decrease of the glucose concentration in the aerobic zone of the

cellulose layer at $c_{S,0}$ = 20 g/l. The simulation shows that no excessive glucose exists in the aerobic zone from the 9th day of cultivation onwards. At this point of time the glucose entering the aerobic zone is consumed instantly. Furthermore, this result validates the conclusion drawn in [4] which implies that the process is limited by diffusion at the latest after 15 days of cultivation.

To validate the model, results of other experiments which were not used for the parameter estimation, were compared to the model simulation. In Fig.2C the calculated and measured layer thicknesses for a reduced glucose concentration are displayed (initial glucose concentration to $c_{S,0}$ = 10 g/l). In this case, experiment and simulation match as well. The situation is different, when the initial glucose concentration in the reservoir is increased in order to create higher driving force for the diffusion: Fig.2D summarizes the results with a starting glucose concentration of $c_{S,0}$ = 40 g/l. While measurements in the initial phase of the cultivation (up to the 25th day) are well predicted by the model, divergences emerge in thickness at the later stages: The real growth of the layer stagnates between the 25th and the 30th day of cultivation, whereas, in the simulation, the cellulose formation continues.

3.5 Discussion

The discrepancies between simulation and experiment in Fig.2D were already observed in chapter 2 under the utilization of the quasi-stationary diffusion model, which is based on the simplifying assumption that the glucose diffusion would be the rate determining effect (compare chapter 2 [4], Fig.9). The dynamic model contains substrate diffusion and growth kinetics as parameters influencing the product formation of the cells. The discrepancy between the simulation results and the experimental data, given by the new dynamic model, after the optimization of the diffusion conditions, affirm the conclusion drawn in chapter 2 [4] which implies that the product formation in the later phases of the cultivation is limited by an effect which is more dominant than the substrate diffusion and cannot be considered exactly in the model. In chapter 4 [7] we suggested that this effect is, in fact, the transport of cellulose from the place of product formation (at the surface-near aerobic cellulose matrix) into the deeper areas of the nutrient broth. In the cited paper it was noted that the growing cellulose is in close contact with the wall of the culture vessel. It is described by the authors that a physical interaction (for instance friction) between the cellulose and the wall leads to a hindered sliding of the cellulose pellet. Such effects are dependent of the material of the culture vessel and on this basis not exactly calculable within an only cell- and product-based model. In chapter 4 [7] we show that an unhindered sliding of the cellulose into deeper zones is essential to maintain the product formation.

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4 EVALUATION OF PRODUCT MOVEMENT INFLUENCES ON THE BIOREACTION

4.1 Abstract

The common way for the production of bacterial cellulose in surface culture is to use culture boxes or beakers with vertical walls, where the maximum achievable thickness is around 4 cm. In order to improve this, it is necessary to study factors limiting the production. In chapters 2 and 3, the mass transfer influences of the substrate combined with growth of bacteria have been investigated. Now we look at a “wall effect”. It is noted that the growing cellulose is in contact with the wall of the box or beaker, and moves downwards into the nutrient broth as time proceeds. Experiments have been carried out where this wall contact was eliminated and a constant rate of production over several weeks was found. This indicates the importance of understanding the role of the wall in the usual surface culture.

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4.2 Introduction

Cellulose, made as an extracellular product from *Gluconacetobacter xylinum* by aerobic surface fermentation, has a higher chemical purity, a significantly higher degree of polymerisation and an improved capacity to absorb water compared to cellulose of plant origin. However, the commercial exploitation of this material has been limited until recently by the unsatisfactory yield of the surface fermentation which needs a lot of parallel batches to be proceed to make sufficient quantities.

The second and third chapter of this work focussed on the influence of substrate mass transfer on the activity of the immobilized bacteria [1]. We had shown that:

- The number of living microorganisms stays constant for a long time after they stopped producing cellulose,
- At the same time as the stagnation of product formation, the substrate supply of the active bacteria becomes diffusion limited. The cells cease the product formation and use the limited substrate only to maintain themselves.
- Increasing the concentration driving force could not totally eliminate the stagnation of product formation.

We showed that the mass transfer limitations of the glucose supply could explain at least part of the observed stagnation of the cellulose growth. Now we attempt a further set of explanation which will involve the movement of the product.

It is important to recollect, that the living cells occupy an oxygen-rich thin top layer of the cellulose. Hence, the previously made cellulose has to move down into the substrate solution. If this sinking process is hindered in any way, this will decrease the formation of new cellulose. In an extreme case, if the walls of the fermenter were not vertical but sloped outwards (see Fig.1A and Fig.4A), only very thin layers of cellulose are formed (< 3 mm). In the conventional vertical walled beakers or boxes, there are interactions between the cellulose layer and the wall, which may hinder the downwards sinking of the cellulose layer. Hence, the interaction between wall and cellulose becomes a highly significant factor which has to be considered in detail. Fig.1B shows the mechanical forces acting on the cellulose layer for the vertical sided flask or box.

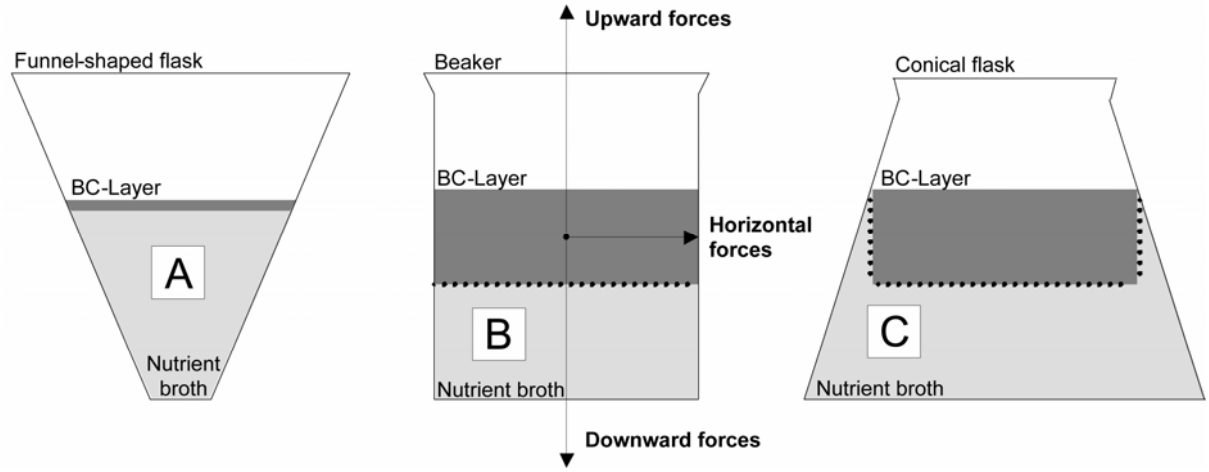

Figure 1. *Different shapes of culture flasks*

Because the cellulose layer is slightly more dense (1030 kg/m^3) than the glucose medium (1008 kg/m^3) there is a downward weight force assisting the sinking movement. In addition, the microscopic and macroscopic roughness of the walls will exert an upwards frictional force whose magnitude is linked to the horizontal forces arising from a slight swelling of the cellulose layer and its adhesion to the wall. The upwards frictional force hinders the downward sinking.

This situation is very complex and, at this stage, an exactly quantifying of all the forces just mentioned, is not possible. However, a series of experiments for an illustration of the relative importance of all these influences (called “wall effect” in the following text) were realized. The evaluation of this wall effect was done by the most simple way: by cultivation of the cellulose forming bacteria in conical flasks (see Fig.1C). These experiments are based at the finding, that during the increase of thickness of a cellulose layer only the active aerobic surface will be reproduced. When a conical flask is used, the layer moves downwards in the liquid and is not in contact to the walls (except for the aerobic surface layer). Thus, the frictional forces mentioned earlier, do not occur. The wall effects are eliminated in conical flasks. This paper shows how the absence of wall effects improves the process yield.

4.3 Materials and Methods

See chapter 2 [1] for a description of these topics. For these experiments, only the vessel details were changed. A 1000 ml conical flask, filled initially with 900 ml of the Schramm-Hestrin medium, was used. An equal surface area for both, the new conical flask and the older vertical beaker, results. Again, this work is based on the natural surface culture described by Schramm and Hestrin in 1954 [2] without any supplements to the media, no regulation or buffering the pH and no genetically modification of the producing strain.

4.4 Results and Discussion

Product formation and bacterial growth

It is discussed in general, that product growth in the vertical walled beakers broadly followed a logistic curve [1]. (For about 6 days there was the exponential phase, followed by another 6 days of linear product formation. Typically after 15 days the growth of cellulose had stagnated.)

When conical flasks are used (which eliminates the wall effect), there is a dramatic increase in productivity, see Fig.2, 3 and 4C. There is a similar duration for the exponential phase, but the linear period continuous without stagnation, as shown by the thickness and the dry mass profile. Thus, this dramatic increase in productivity has been clearly demonstrated for our strain of micro-organism. (Other strains were not tested.)

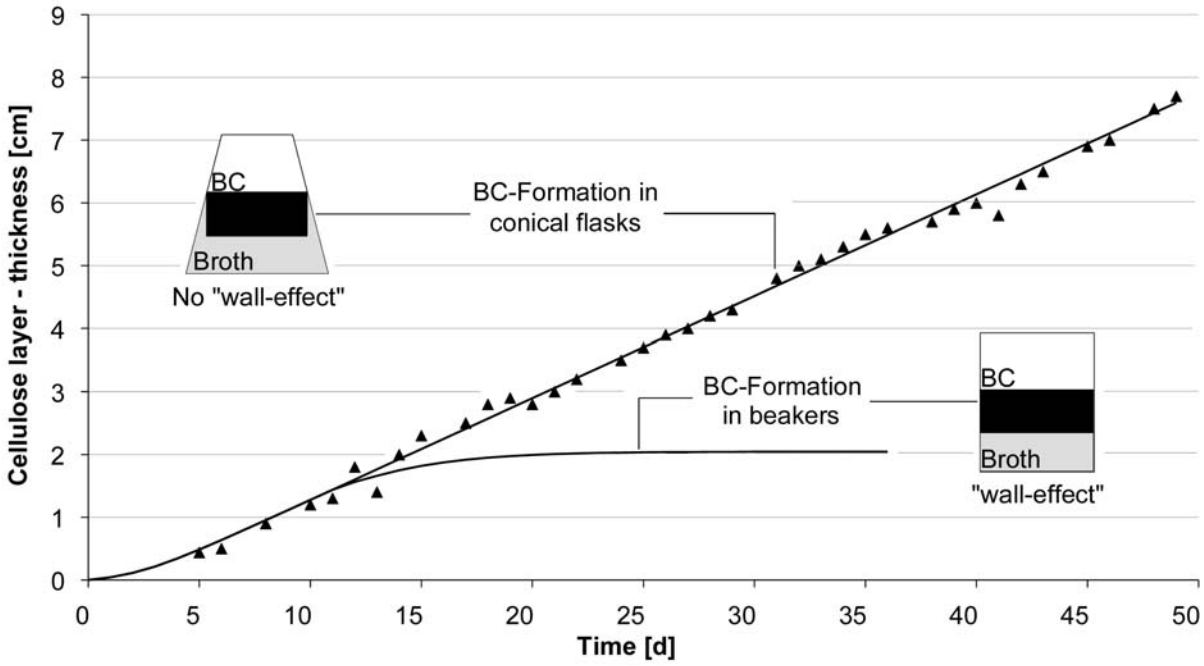

Figure 2. *BC layer thickness after eliminating of the "wall effect" (from 37 independent measurements) in comparison with the BC formation in vertically walled beakers*

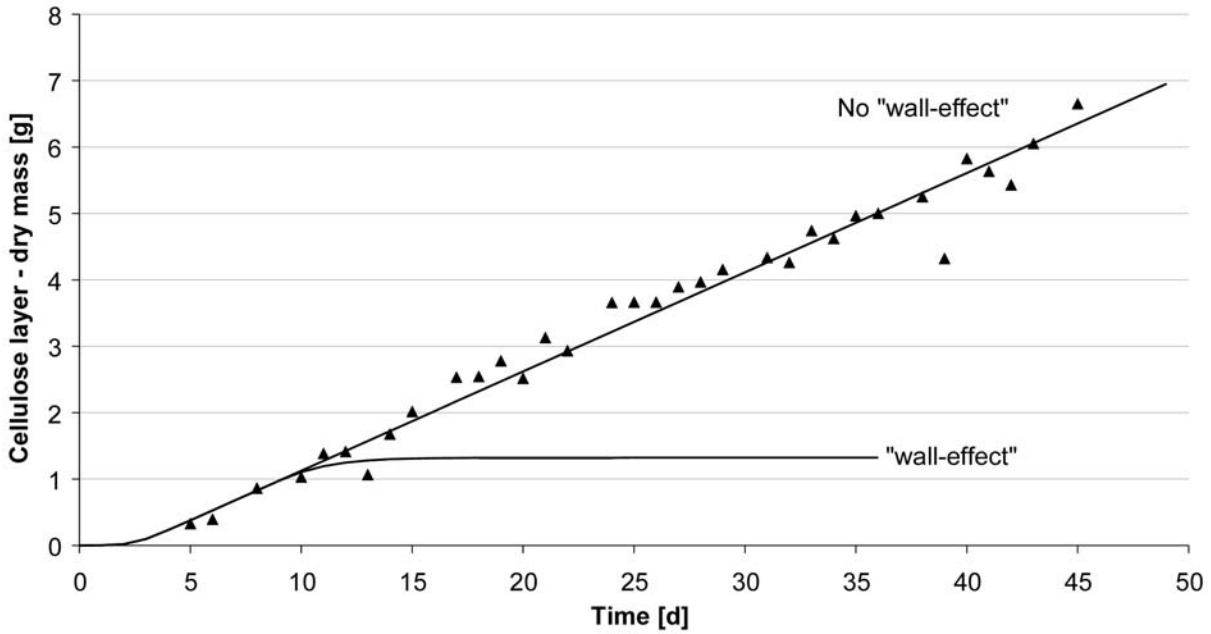

Figure 3. *BC layer dry mass after eliminating of the "wall effect" (from 34 independent measurements) in comparison with BC formation in beakers*

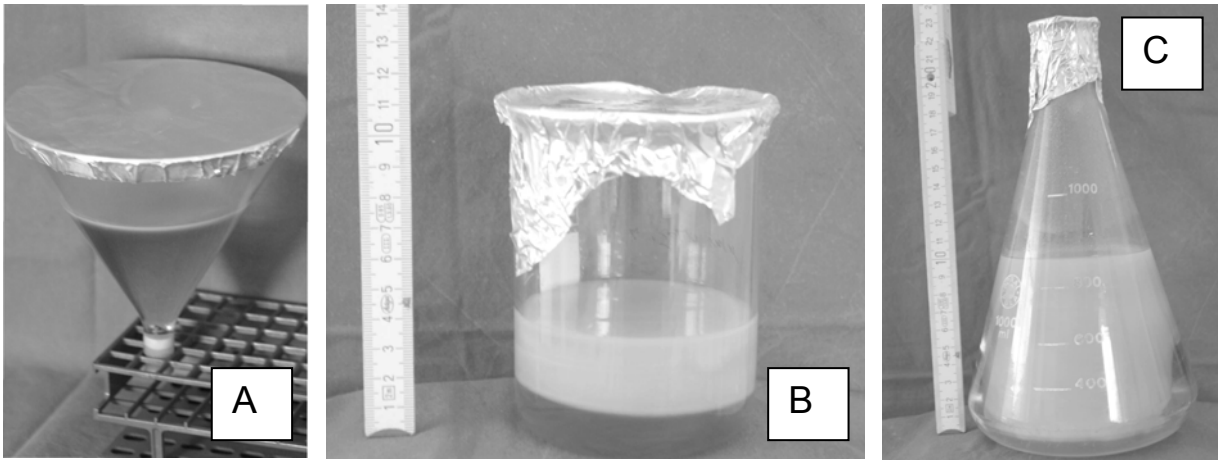

Figure 4. *A: max. 3mm thick BC layer produced in a funnel shaped flask, B: max. 2 cm thick layer produced in a beaker, C: 10 cm thick BC layer produced in a conical flask*

We can fit a linear regression line through the data of Fig.3 (ignoring the exponential phase which occurs in the first six days). It was found that:

$$P_{BC,dry\,mass}(t) = 0,1644\frac{g}{d}\cdot t - 0,2727g \quad \text{(for t > 6 days, } r^2 = 0,99) \qquad (1)$$

Fig.5 clearly shows that the cell density is the same for the cellulose made in both, beakers and conical flasks. This observation results from the fact, that the maximum cell density is limited by the

space provided within the matrix of cellulose fibres. The densities of fibres are similar from both types of fermenter vessels (confirmed by comparative measurements of percentage cellulose dry mass). The total number of cells in the figure was calculated from the cell density results, plus data from Fig.2.

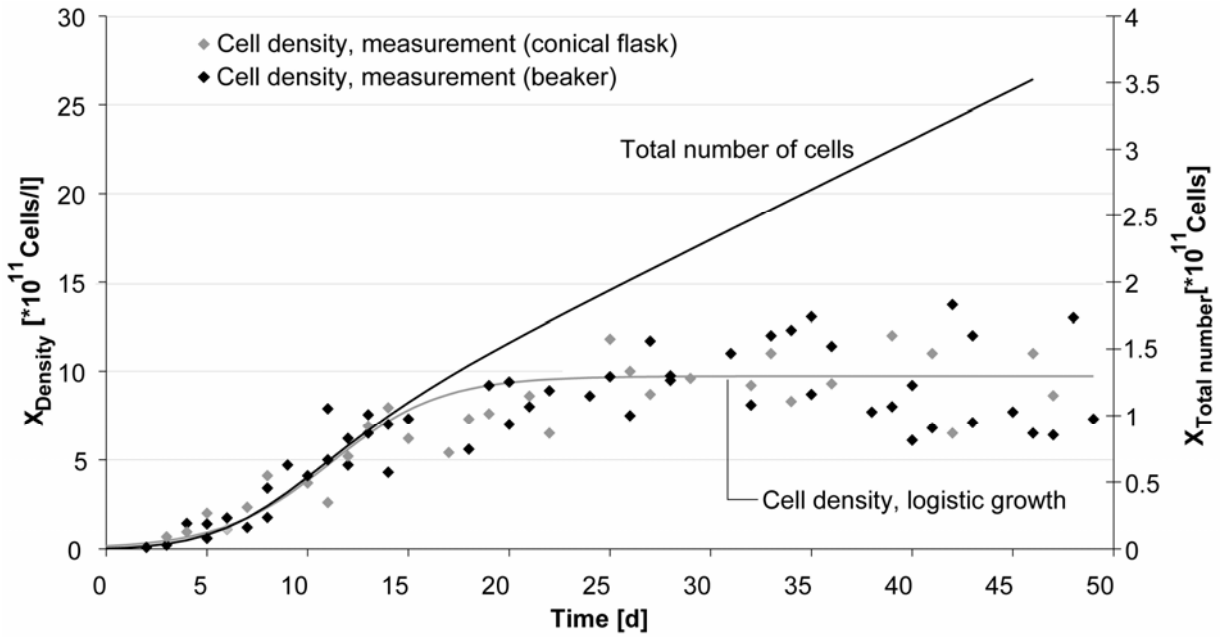

Figure 5. *Cell density (beaker and conical flask) and absolute number of cells within a cellulose layer (conical flask)*

The course of total number of cells within a conical flask cellulose layer can be approximated as a linear form according to equation (2) after a six days adaptation time.

$$X_{TC}(t) = 8,3 \cdot 10^{9} \frac{\text{cells}}{\text{d}} \cdot t - 4,6 \cdot 10^{10} \text{ cells} \quad \text{(for t > 6 days)} \qquad (2)$$

In chapter 2 [1] it is described, that the living bacteria exist at the upper cellulose layer which has an adequate oxygen supply. The number of this cells were estimated to be $8,3 \cdot 10^{9}$ cells (see Fig.6 in chapter 2 [1]) for the beaker experiments.

In the case of conical flask data, which there are just discussed, equation (2) relates the total cell number to time (after the initial 6 days period). The active cell number equals the gradient of this straight-line graph which is $8,3 \cdot 10^{9}$ cells (relating to 1 day). Note that this is the same quantity as in the beaker experiments (see chapter 2 [1], Fig.6). However, this same number of active cells are far more productive in the conical flask environment where there is no wall effect. This constant productivity was calculated to be $1,8 \cdot 10^{-11}$ g dry mass per cell per day or $1,9 \cdot 10^{-11}$ mm BC thickness per cell per day. (In the previous beaker experiments, these figures were obtained in the earlier part of the fermentation from days 6 to 10 but soon reduced, eventually stagnating.)

Substrate consumption of the bacteria

In a similar way to chapter 2 [1], there was monitored again the glucose consumption of the bacteria within the aerobic zone. Fig.6 shows the measured dynamics of glucose assimilation and a simulation of the glucose assimilation using equation (3) which describes the consumption of glucose for cell growth, product formation and viable cell maintenance.

$$S(t) = \underbrace{Y_{\frac{S}{X_{TC}}} \cdot X_{TC}(t)}_{\text{(Growth of total cells, TC)}} + \underbrace{Y_{\frac{S}{P}} \cdot P(t)}_{\text{(Product formation)}} + \underbrace{k_e \cdot \int_0^t X_{VC}(t)dt}_{\text{(Maintenance of viable cells, VC)}} \quad (3)$$

We adapt equation (3) by substituting the two linear kinetic equation for growth of the total number of immobilized bacteria [Fig.5, total cell number and equation (2)] and the product formation [Fig.3, BC-dry mass and equation (1)] and the integrated form of the constant number of viable cells (X_{VC} = $8{,}3 \cdot 10^9$ cells·days). For the yield coefficients $Y_{S/X_{TC}}$ and the maintenance rate k_e, we used again the values determined in part I ($Y_{S/X_{TC}}$ = $1{,}22 \cdot 10^{-11}$ g glucose per cell; k_e = $3{,}86 \cdot 10^{-12}$ g glucose per cell and day). Fig.6 shows the result of the simulation.

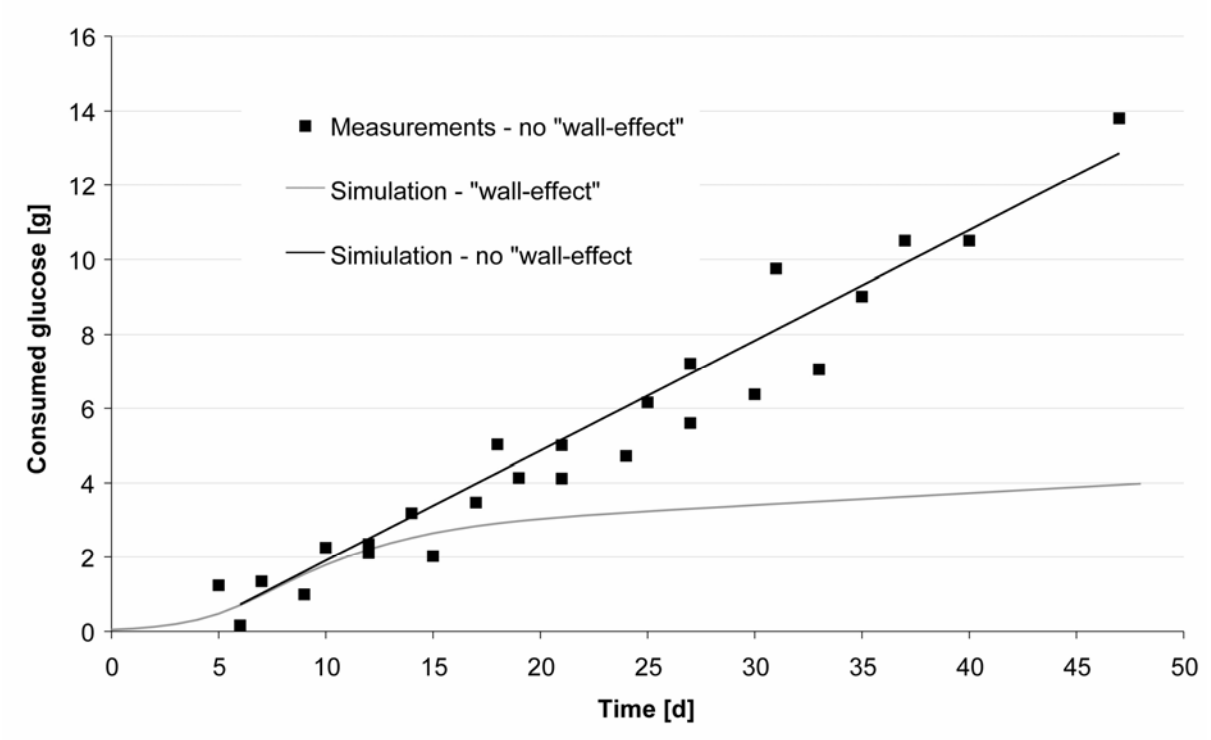

Figure 6. *Glucose consumption in a conical flask (from 25 independent measurements) in comparison with the wall effected BC formation in beakers*

The calculated profile of the substrate consumption shows a good correlation to the measurements. For the conical flask experiments, a noticeable linear trend is evident in the glucose consumption. This is expected as we have already shown the linear nature of cell growth and BC production. In contrast

to the conical flask experiments, product formation and increasing of the total number of cells in beakers becomes hindered between day 15 and 20 of the fermentation. From this point, only a relatively small amount of glucose is needed for cell maintenance (small rise of the grey line in Fig.6). The results of part two shows that the stagnation of the product formation is mainly due to the wall effect. For the beaker experiments, mass transfer limitations also found to be evident.

Such limitations are removed in the conical flask fermentations. Just to clarify this question, we can now extend the previous model to describe this new situation.

Modified Glucose Diffusion model

It is necessary to adapt the diffusion model to the conical flask conditions [3]. In the vertical wall experiments, the diffusional area is constant, whereas in a conical flask, the area increases as the vertical cylinder of cellulose progressively moves down into the substrate layer (see Fig.1B and 1C, dashed lines). Hence we need to add an extra equation into the model to represent this time varying feature:

$$A_{Diff}(t) = \frac{\pi d^2}{4} + \pi d \cdot y(t) \tag{4}$$

where d is the diameter of the cellulose layer. Tab.1 gives the parameters, which are the same as in chapter 2 [1] (note that the diameter of the conical flask was chosen to be the same as the beaker).

Parameter		Value and unit	
d	Diameter of cellulose layer	0,072	m
$C_{S,0}$	Initial substrate reservoir Glucose concentration	20	kg/m^3
D	Diffusion coefficient of glucose in cellulose	$4{,}05 \cdot 10^{-10}$	m^2/s
m	Equilibrium constant	1	---
$Y_{P/S}$	Yield coefficient (glucose converted into cellulose)	45	%
$\rho_{BC,dry}$	Dry cellulose fraction	0,01	---
$V_{S,0}$	Initial substrate reservoir volume	0,001	m^3
ρ_{BC}	Density of cellulose	1030	kg/m^3
ρ_{Water}	Density of water	1000	kg/m^3
$\rho_{Glucose}$	Density of glucose	1550	kg/m^3

Table 1. *Parameters for simulation of glucose diffusion*

We can solve equation (9c), (10a) and (11) from chapter 2 together with the new equation (4).

Clearly, this theoretical model is an approximation to the true situation which should be modelled by a partial differential equation to show the glucose concentration as function of depth and radial position in the existing cellulose film. However, the reasonable accuracy of the predictions shown below suggests that this level of sophistication is not required to explain the observed growth rates, see Fig.7 (black curve).

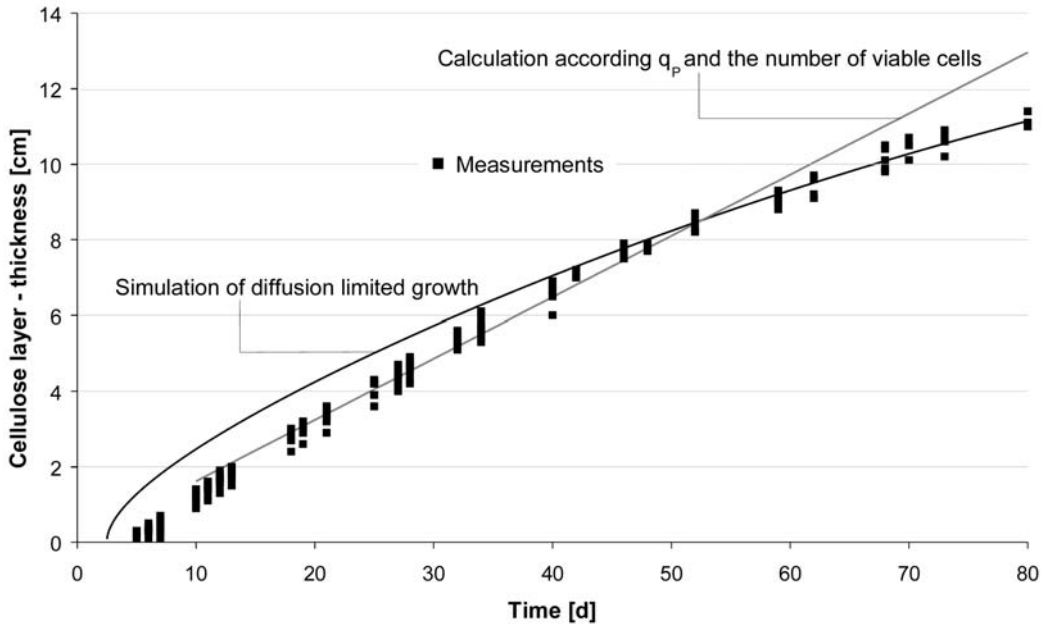

Figure 7. *Thickness during a long term experiment: comparison of experimental data, prediction using the diffusion model and a calculation using the specific rate of product formation of the viable cells*

Fig.7 shows the experimental measurements, predictions from the modified diffusion model and a further set of predictions using the information on the constant cell productivity results from the conical flask experiments. The formation of BC after eliminating the wall effect in the early stage is well described by the productivity model. Due to the constant number of living cells and the constant productivity of these cells, we have a linear increase of the product in the initial stage (see Fig.7 grey curve). Whereas the diffusion model overestimates the product growth from start to day 50 of fermentation but is pleasingly accurate later on. Clearly, the rate determining step up to the 50th day (where the two sets of predictions intersect) is the microbial kinetics, but after this time, the cellulose layer is sufficient thick (and glucose concentration has decreased) for the rate of glucose diffusion to become the limiting step. (In chapter 2, there was a similar effect in the beaker experiments, where at low times, the consumption of glucose was over estimated).

One unexpected observation was made with these longer fermentations. The enrichment of by-products of the metabolism in the substrate reservoir and in the cellulose layer which reduced the quality of the cellulose formed in later stages of the fermentation.

Up to now, it was discussed vertical and conical walled fermenter vessels in detail. We can envisage other shapes of containers for further explanation of the hindering effect.

Comparing diffusion and wall effects

As shown, there is no wall effect in a conical flask and the area for diffusion increases with time. In a beaker, the area is constant but there is a definite effect to hinder the observed growth rates.

A new arrangement is shown in Fig.8, where a conical flask has a glass stick placed in the centre. The stick introduces the wall effect into this modified conical flask. The cellulose will grow in an annular shape into the substrate. The geometry was chosen to give the same BC surface in both containers and the same wall circumference. The results showed that the thickness of the annular shaped cellulose layer stagnated at around 3 to 3,5 cm (compared to approximately 2–3 cm in the usual vertical walled beaker).

Hence, in this instance, the wall effect outweighs the benefit of an increasing diffusional area.

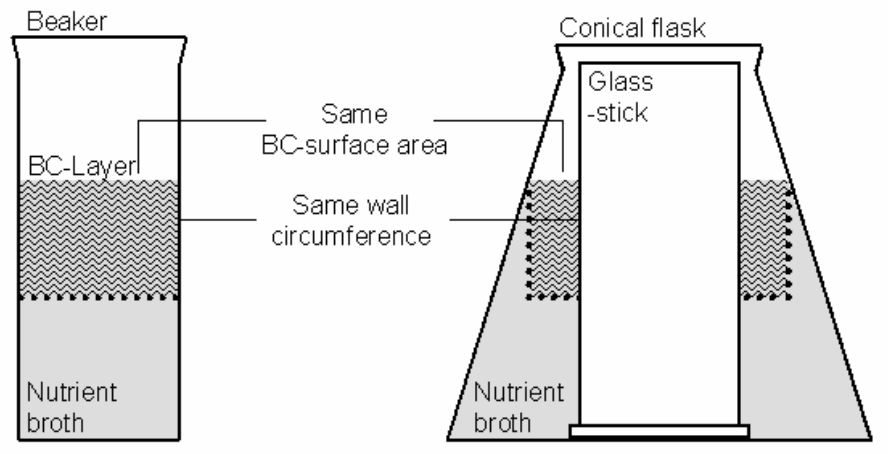

Figure 8. *The modified conical flask experiment*

The morphology of cellulose formed in conical flasks

One interesting point, which was not examined in this work, considers if the elimination of the contact between the wall and the cellulose could effect the morphological structure of the cellulose and its density. At the moment, this is being studied and the results will be published in due course.

4.5 Conclusion

This investigation into the static cellulose surface culture confirms the assumption that external factors limit the product yield. Therefore, it becomes clear that a process optimisation exclusively focused on the enhancement of the microbial conversion of the carbon source into bacterial cellulose (which were described often in the past) are unsuitable to achieve a fundamental improvement. The results of this work clearly identify the external factors which limit the production of bacterial cellulose in surface culture: namely the wall effect and less important glucose diffusion. In addition, when we improve the process it allows long production runs. There is the possibility that by-products can be enriched in the substrate reservoir or in the product. Clearly, this has a negative influence and needs to be minimized.

To improve the process we need:

1. The elimination of the wall effect, which is the strongest limiting factor.
2. The prevention of substrate limitation, which may reduce the capacity of the bacteria, especially in later stages of the fermentation.
3. The prevention of by-product enrichment in the medium reservoir or in the product.

In a following chapter the design of an apparatus, fulfilling all these requirements, is discussed.

4.6 Appendix

Influence of wall roughness and wall material

As it is shown, the wall of a fermenter vessel can have a major effect on the course of the production of microbial cellulose. Up to now, we have only used glass vessels. The table below summarises some data where different materials were used for the beaker. The approximate surface roughness figures are given, together with the maximum cellulose thickness made in these beakers.

Vessel material	Surface roughness, R_a	Maximum thickness
Duranglass	< 10 nm	approx. 20 mm
Polished steel	< 0,8 µm	approx. 12 mm
Polypropylene	0,5–3,2 µm	approx. 7 mm

Table 2. *Influence of surface roughness on maximum BC thickness*

Broadly speaking, the table shows the smoother the surface, the higher the maximum thickness made in these beakers. (Clearly the influence of contact angle has been ignored here.) In addition, the surface's chemical characteristics such as hydrophobility may also have an effect on the formation of cellulose in vertical walled beakers. Experiments are being carried out currently to investigate this further.

Acknowledgements

The authors are grateful for grants from the German Ministry of Economics and Labour as well as the Thuringian Ministry of Economics, Labour and Technology and for grants from the European Union (LEONARDO programme).

4.7 References

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[2] M. Schramm, S. Hestrin, Factors affecting Production of Cellulose at the Air/Liquid Interface of a Culture of Acetobacter xylinum, J. Gen. Microbiol. 1954, 11 ,123-129

[3] A.M. Gerrard, M. Hornung, H.P. Schmauder, Numerical Modelling of Bacterial cellulose manufacture, 7th World Congr. of Chem. Eng. - Congress Manuscripts, 2005

5 A NOVEL AEROSOL BIOREACTOR WORKING ON A FED BATCH PRINCIPLE

5.1 Abstract

In chapters 2, 3 and 4 it was shown that the rate of production of bacterial cellulose stagnates because of the limitation of substrate supply and a wall effect, which hinders the removal of the product from the active cell zone. The following chapter demonstrates, how both of these problems can be eliminated in a novel bioreactor, where the substrates (mainly glucose and oxygen) are both fed directly to the surface of the product cellulose. This involves the generation of an aerosol spray of glucose and its even distribution to the living bacteria on the medium-air interface. The apparatus was built and operated up to eight weeks with a constant rate of cellulose production. The aerosol system provides the basis for an economic production of bacterial cellulose in surface culture.

This chapter have been published in *Engineering in Life Sciences:*

M. Hornung, M. Ludwig, A.M. Gerrard, H.P. Schmauder, Optimising the Production of Bacterial Cellulose in Surface Culture, Part III: A novel aerosol bioreactor working on a fed batch principle, *Eng. Life Sci.* 2006 7 (1), 35-41

5.2 Introduction

In previous chapters it was described how bacterial cellulose can be produced in a surface fermentation [1, 2]. The process was also described mathematically. The use of vertical sided vessels led to problems of mass transfer limitation of the glucose supply and a wall effect which caused the production rate of cellulose to a stagnation. This paper describes the development of a novel system which circumvents these difficulties.

In other previous papers, insufficient substrate supply to the bacteria was suspected to be the external factor limiting the yield of the surface culture. These considerations resulted in special types of horizontal fermenters being developed, where the polymer is deposited on the surface of a drum [3] or disks [4, 5] rotating around the long axis. Part of the drum or disk surface temporarily dips in the liquid medium, the remainder is above the surface in the air. The advantage of this method is: bacteria on the disk has good contact with both the air and the glucose solution. The rotating disk reactor of Serafica et al. [5] produced 1 g cellulose (dry mass) after 5 days of growth. Sattler [3] reports a 5 mm thickness of cellulose layer after 8 days of fermentation. A third example from Krystynowicz [4] which gave 12,6 g dry mass in 7 days. A further development of these devises is not described yet. But on the basis of these figures, the production rates are still relatively low. Our experiments with an self constructed rotating disk system gave similarly disappointing throughput figures. In addition, the quality of bacterial cellulose was low. In particular, the cellulose was not homogenous. Some sections were mechanically weaker than others. Another problem involved the growth on unwanted cellulose in the substrate reservoir, which interfered with the movement of the rotating disk. In addition, as the desired cellulose layer became thicker, it tended to separate from the metallic mesh on which it was supposed to form.

The results from chapters 2, 3 and 4 [1, 2, 9] and the rotating disk experiments, lead us to suggest the following criteria for a successful development of a higher capacity system:

1. Eliminating of the wall effect, which has been shown the strongest limiting factor.
2. Prevention of substrate limitation which may limit the capacity of the bacteria in a later stage of incubation.
3. Prevention of any enrichment of by-products within the substrate reservoir.
4. Prevention of any movement of the cellulose layer or flowing of liquid medium across it.

In order to design a new process to achieve the above requirements, the key issue is the reversal of the direction by which the glucose reaches the living bacteria (see Fig.1). The left hand diagram shows the product having to move bodily downward away from aerobic growth zone (see Fig.1, left hand, note the position of a tracer at the beginning and end). This involves the wall effect. The glucose has to pass through the increasing cellulose layer. In addition, the reservoir conditions are optimal

only at the start of the growth period, thereafter the glucose concentration changes, as does the pH and by-products become enriched.

In contrast, the right hand sketch shows that both the oxygen and the glucose are fed from the top. This direct feeding of the glucose produces a thin fluid film above the active bacteria. The bacteria penetrate the extended interface and produce new cellulose fibres at this location, ready for the next fluid film of glucose. The product does not need to be transported downwards, because the aerobic zone moves upward. A tracer segment (see Fig.1, right hand) does not change position during the fermentation. The diffusion of glucose through the cellulose is involved and the composition of the glucose feed can be maintained at a constant value.

The principle of direct substrate feeding has been in use for a number of years to culture plant tissues such as callus, roots or shoots. A detailed review of these topics is given by Weathers and Zobel [6].

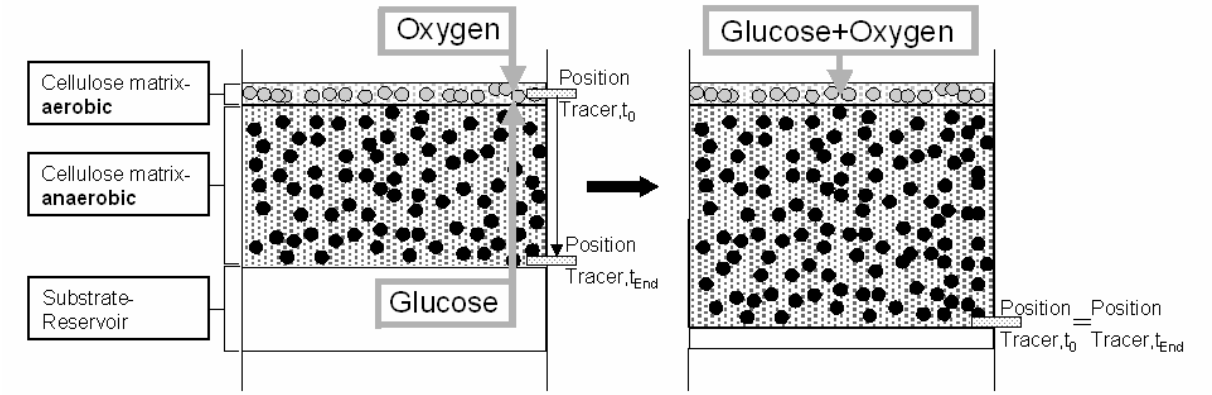

Figure 1. *Changing from an indirect to a direct feeding of both substrates (glucose and oxygen)*

5.3 Materials and Methods

Supply the substrate directly

Product quality is an absolute requirement in the development of the new fermenter. The cellulose layer must be mechanically homogenous, and all the material must be equally strong. This means that we cannot allow puddles and large drops of nutrient broth to form on the top surface, so the application of the fluid has to be consistent and controllable. The rate of input of the substrate to the growing layer has to equal the rate at which microorganisms are utilising the glucose. The most efficient and practical way of achieving this aim is to use an aerosol to spray the glucose onto the active top surface of the cellulose layer, see Fig.2.

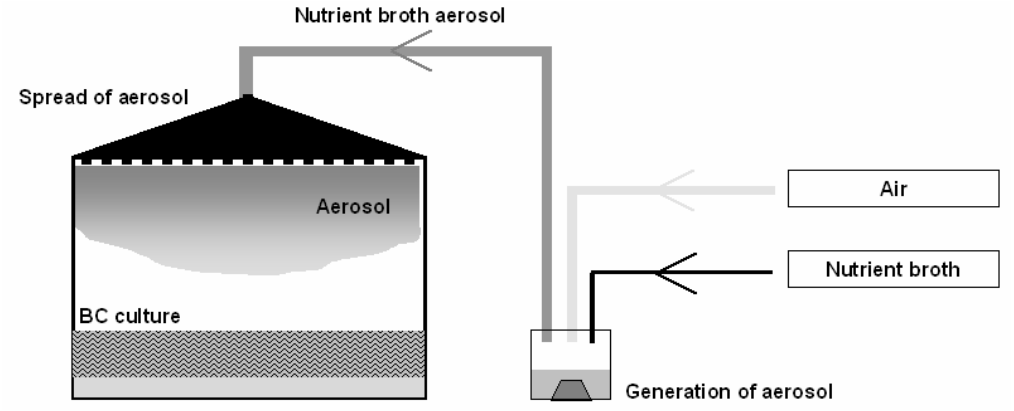

Figure 2. *Direct supply of substrate by an aerosol*

Here, the sterile nutrient broth and sterile air are fed to a separate aerosol generator which creates a spray which is transferred to the head space at the top of the fermenter. It is then evenly distributed and settled on the top layer of the cellulose where the bioreaction occurs. The initial experiments were done with a simple aerosol reactor, as in Fig.2. The promising results obtained with this simple arrangement encouraged us to scale-up and extensively optimise the simple aerosol reactor. A fully developed rig has been built in our laboratory [7] and is shown in Fig.3.

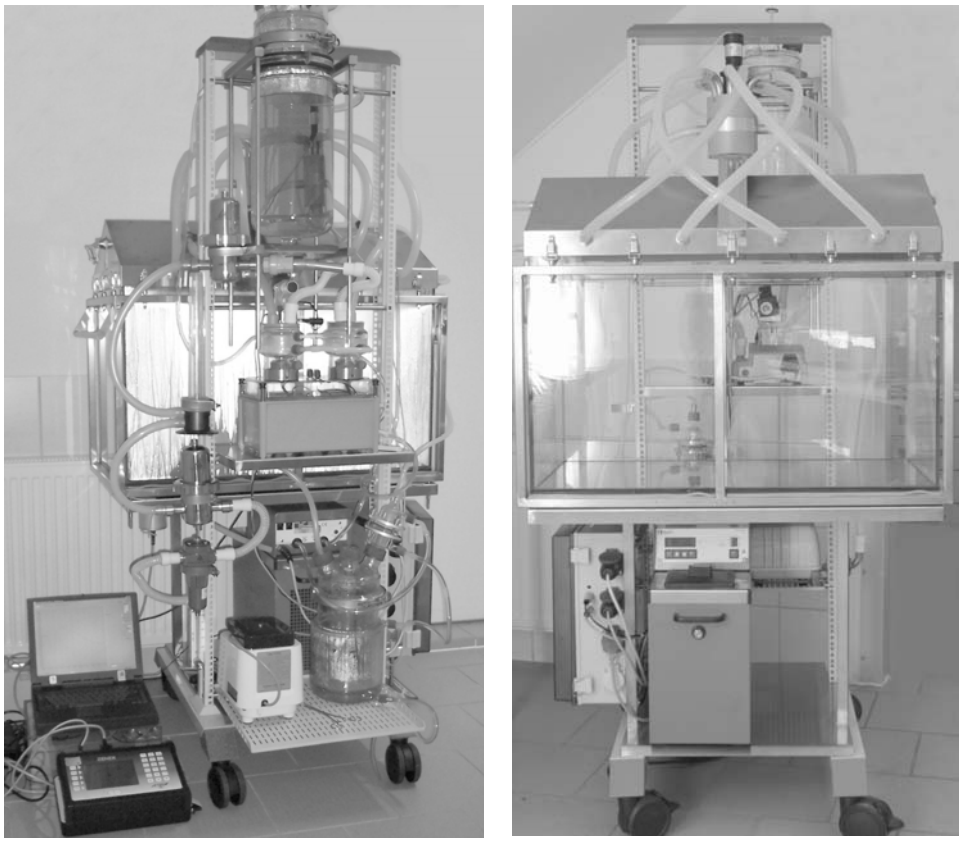

Figure 3. *The aerosol bioreactor (left hand: back; right hand: front)*

Generation of substrate aerosol

The aerosol generator is shown below (Fig.4, left hand) produces the spray by ultra sound.

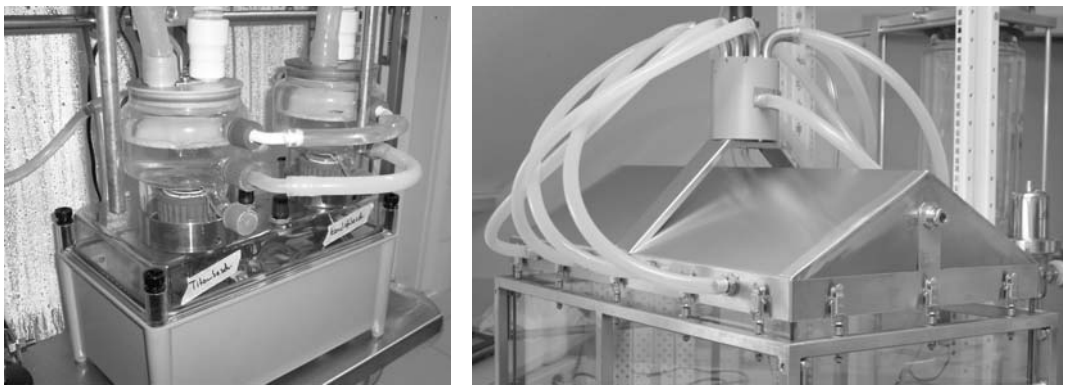

Figure 4. *The substrate aerosol generator (left) and aerosol spreading (right)*

The conversion of the liquid into an aerosol takes place in two removable (and sterilisable) chambers (see Fig.4, left hand). They are filled by gravity from a feed vessel above these chambers and the level is automatically controlled. Special treated piezo-electric crystals are used as the vibration source (at 1,68 MHz). It is important to provide cooling for the electrical system which powers the vibrations, otherwise the reliability of the system is poor. The sterile air (1200 l/h, 0,2 bar_G) is introduced and the spray is transported into the aerosol distribution box. (The droplets range from 0,5 to 6 µm, with 85 % < 4 µm.) The maximum liquid flow rate is 10 ml/min.

Homogenous distribution of the substrate aerosol

There is an absolute necessity for a homogenous distribution of the aerosol to the total cellulose surface. To achieve this, we designed and built a distributor which had eight channels (Fig.4, right hand). The spray is fed to a rotating disk device (4 rpm) which feeds two out of the eight exit channels at any one time. There are flexible tubes (20 mm inner diameter) which then lead the spray to the special roof-shaped distribution box situated on top of the culture box. The two streams come into the vessel horizontally, on opposite sides, at the same time. This causes much turbulence which ensures good mixing in that section of the culture box. A few seconds later, a different pair of channels is selected, which feed spray into another part of the aerosol box. Any large droplets (arising from coalescence of smaller droplets) which touch the inner surface of the distribution box remain attached to the sloping roof and flow down into a open channel and are removed from the system. This arrangement ensures the even and steady supply of substrate solution along with the air, to the living bacteria on the cellulose surface.

Culture box

The culture box (500 mm x 900 mm x 500 mm) is made of temperature resistant security glass (thickness 10 mm). The edges are sealed with special silicone which has been shown to be biocompatible. This box, the distributor and the aerosol generator operate under a small positive pressure to ensure that no stray organisms enter from outside.

Sterilisation

The sterilisation of the culture box and distribution lid is done using hot air at 180°C for 2 hours. The aerosol chambers, the receiving vessel for condensed substrate and the reservoir are autoclaved for 30 min with 121°C steam.

Temperatures

In order to keep the liquids sterile for as long as possible, the contents of the aerosol generator, substrate reservoir and receiving vessel are kept at 3-4°C (by circular refrigerated cooling water).

Measurement of cellulose thickness

The thickness of the cellulose layer was measured daily with a ruler from the outside of the culture box. With regard to future automation of the aerosol bioreactor, we established a second method for on-line thickness measurement of the cellulose layer (cellulose thickness is the main controlled magnitude). Therefore we chose a reflection based microwave sensor with regard to the high water content of the cellulose layer (approx. 99 %). The dielectric permittivity of water is approximately 80 whereas the dielectric permittivity of air is one. In consequence of the sharp spatial separation of the cellulose layer from the air, it is possible to measure the increase of the thickness of the cellulose layer by measurement of the dielectric permittivity. The core element of the set-up was a measuring head (patch-antenna with electronics, PC-controlled), which was pressed against the glass bottom of the culture box without direct contact between cellulose and antenna. The usable frequency range was 2,2–2,65 GHz with a resolution of 80 kHz. Over the antenna a electromagnetic wave was radiated into the cellulose. The reflection factor was determined by division of the measured amplitudes of the initial wave by the amplitudes of reflected wave within the frequency range. The sweep for this range required less then one second. The conversion of the obtained data into a cellulose thickness was done by a PC and a special calibration routine.

Measurements of chemical and physical properties of cellulose

Tensile strength of wet cellulose membranes have been measured using a ZWICK 1445 mechanical tester. It is given the force (F_{max}) which is necessary to break a cellulose membrane (after slicing and washing, dimensions of about 0,1 cm thick, 4 cm wide, 20 cm long).

The mechanical tightness of the bacterial cellulose was evaluated using the Texture Analyser (TA-XT 2i, Stable Micro Systems). A punch (1 cm^2) was placed on the surface of the cellulose layer (direct from the culture box; dimensions approx. 4 cm thick, 10 cm wide, 10 cm long) and pressed in for 10 mm with constant speed. The load-displacement relation was measured and used to characterise the resistance of the cellulose.

The degree of polymerisation of bacterial cellulose was determined by capillary viscometrie (PVS1, Lauda with a 0,004 mm Micro-Ostwald-capillary) after freeze dried cellulose has been milled and dissolved in copper(II)ethylenamine solution.

SEM investigation

Wet membranes of bacterial cellulose were dried by lyophilisation and mounted on stubs for SEM. After covering with gold using a sputter coating device BAL-TEC SCD005 (Balzers, Lichtenstein; 60 mA, 80 s, gold coating approx. 35 nm), the films were studied with a LEO-1450 VP (LEO,

Oberkochen, Germany) scanning electron microscope operating at 15.00 kV. The micrographs were taken at a magnitude of 8.00k X and a working distance of 8 mm, 10 mm and 11 mm, respectively.

Microorganism

In all experiments, the wild type strain *Gluconacetobacter xylinus* from the stock collection of the Research Centre for Medical Technology and Biotechnology (Geranienweg 7, D-99947 Bad Langensalza, Germany, phone:+49-3603-833-145, fax:+49-3603-833-150, e-mail: forschungszentrum@fzmb.de) was used.

Experimental fermentation and media

The classical Schramm/Hestrin (SH) media with the following composition per litre was used: 20 g Glucose; 5 g BactoYeast Extract; 5 g BactoPeptone; 6,8 g $Na_2HPO_4x12H_2O$; 1,115 g Citric Acid. All media were autoclaved for 20 min at 121°C. The glucose solution was autoclaved separately and added aseptically after the media had cooled below 50°C.

5.4 Results

Product formation and properties of the product

The experiment is started by putting 1 l of inoculum into the culture box and waiting for the cellulose layer to form (up to 5 days). Then the aerosol is gradually introduced in stages to allow the bacteria to adapt to the new conditions. This takes about one week. At this point, the aerosol is fed in for 5 minutes then switched off for 5 minutes and this pattern continues. Fig.5 shows that we then get a period of linear growth which can last for up to 6 weeks. This run was stopped for unsterility reasons. The average growth was 2 mm/d or around 9 g cellulose dry mass/day).

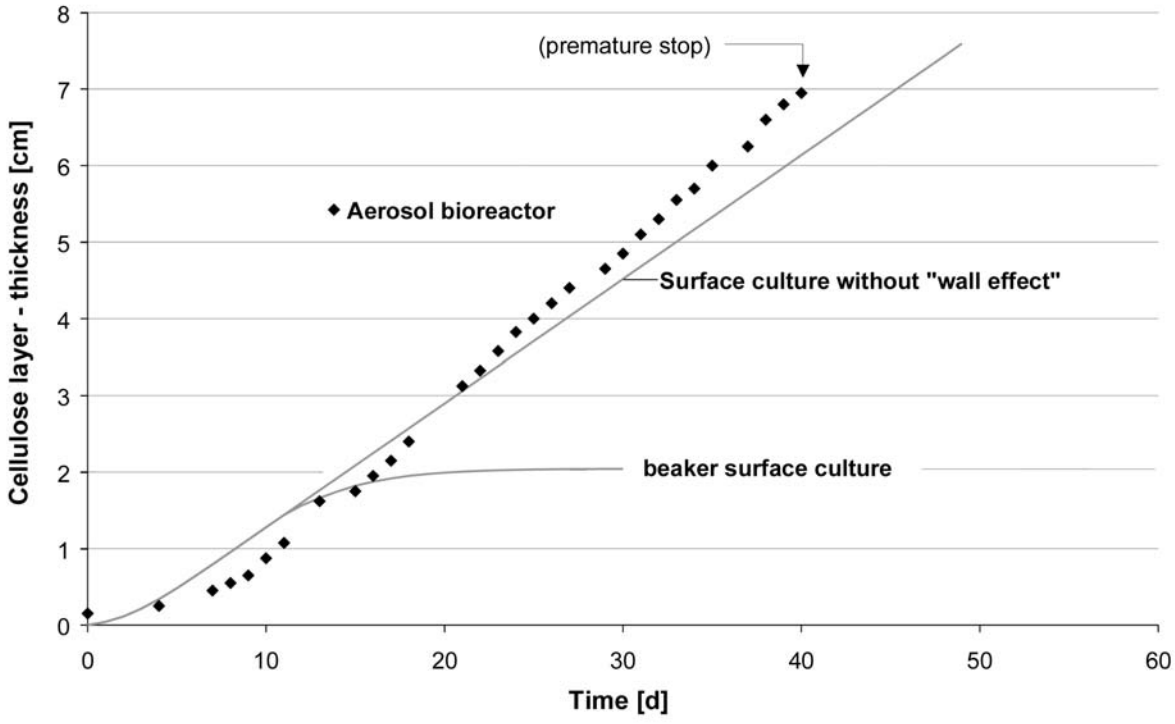

Figure 5. *BC pellicle thickness produced in the aerosol bioreactor compared to the beaker and conical flask process*

The smooth and even distribution of the aerosol on the cellulose layer leads to a product with higher quality than that obtained by the static surface culture. This can be demonstrated Tab.1.

Characteristic	BC produced in the aerosol bioreactor	BC produced in usual surface culture
Tensile strength, F_{max}	114 N	45 N
Gradient of graph in load-displacement diagram	34,7 N / 10 mm	8,9 N / 10 mm
Degree of polymerisation	5200	9900

Table 1. *Characteristics of BC produced in usual surface culture and aerosol bioreactor (every value is the average from 5 independent measurements)*

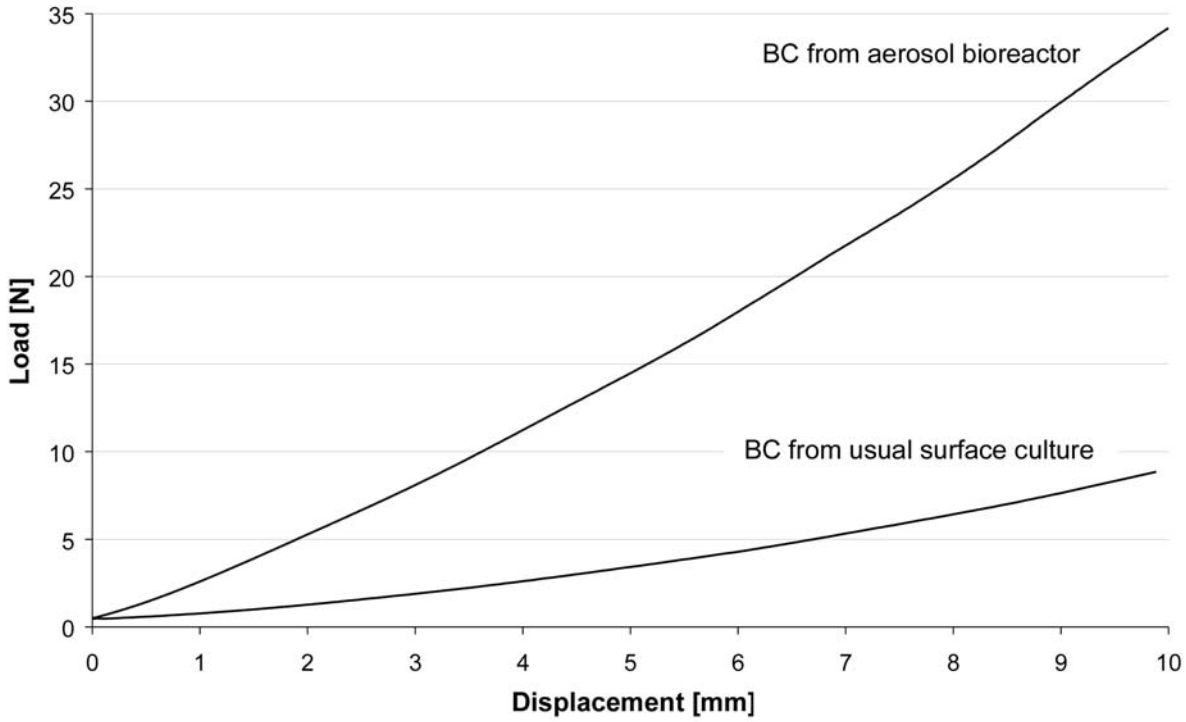

Figure 6. *Load-displacement curves of BC from aerosol bioreactor and BC from static surface culture*

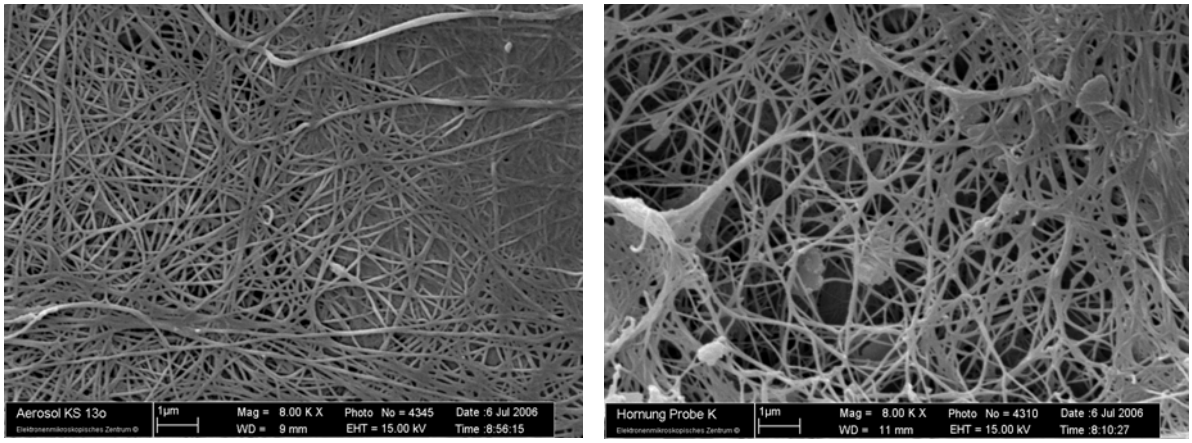

Figure 7. *Scanning Electron Microscopy of BC from aerosol bioreactor (left) and BC from static surface culture (right)*

BC formed in the aerosol bioreactor gave the haptic impression of having a higher mechanical strength than the traditionally produced product from a static beaker or box culture. This could be verified by measuring load-displacement curves. Whereas a load of 8,9 N is sufficient to press a 1 cm^2 stamp 10 mm in a usually produced cellulose layer, the load has to be about four times higher if the cellulose was produced in the aerosol bioreactor (Tab.1, Fig.6).

Membranes of bacterial cellulose have a wide range of applications. For this reason we measured the tensile strength of cellulose membranes, produced in the aerosol bioreactor in comparison with cellulose membranes, produced in usual surface culture. These values (see Tab.1) shows a clear difference. The force to break an aerosol bioreactor cellulose membrane (F_{max} = 114 N) is more then doubled compared to usual produced cellulose (F_{max} = 45 N).

The microscopic analyses of cellulose membranes, produced in the aerosol bioreactor (Fig.7, left) shows that aerosol process does not adversely affect the bacteria. The fibre-network is seen to be even and without damaged fibrils. Compared to the membranes produced in usual surface culture, (Fig.7, right) the network of fibrils of the aerosol bioreactor appears to be more dense. Possibly the higher mechanical quality of the “aerosol cellulose” is caused by this denser network.

The degree of polymerisation (DP) of cellulose, which was produced in the aerosol bioreactor is lower then the DP of usual produced cellulose (see Tab.1). A cellulose molecule formed in the aerosol bioreactor consists of approx. 5200 glucose units (approx. 9900, if produced in usual surface culture). The lower DP of the cellulose from the aerosol bioreactor probably results from the aerosol’s mechanical disruption of the polymerisation reaction. However, the results mentioned above clearly show, that the lower DP of cellulose, formed in the aerosol bioreactor, has no negative influence on the mechanical properties of the product.

Formation of slices of BC

When the cellulose layer is to be harvested from the aerosol bioreactor, the distributor box is removed and the block of product is lifted out by hand. To make this operation easier, some experiments were carried out with the objective of being able to lift out the cellulose, not as one thick layer, but as a number of slices. The strategy was to switch of the supply of substrate for a period of time before

restoring the normal operating conditions. Time intervals from 2 to 24 hours were used. The best results were found when a 6 hour interruption was used. This allowed the cellulose to be peeled off, as a number of 3–4 cm thick layers (see Fig.8). The short starvation period had no negative effect on the product quality.

Figure 8. *Formation of slices by interrupting the aerosol feeding*

The aerosol bioreactor is the result of the first phase of development. The one negative aspect of this initial version of the bioreactor concerns the ingress of contaminating organisms. This usually occurs between 6–10 weeks after the start of fermentation. The location of the problem is usually in the aerosol generator or the distribution box, which cannot be intermediary disinfected during a production run. It is not known if the contaminating species come from outside the apparatus or arise from an imperfect sterilisation of the components at the start of the process. In Fig.5 the premature stop at around 40 days was caused by this problem.

5.5 Discussion

This new bioreactor was developed to eliminate problems which were inherent in the older beaker experiments, including the hindering of the growth by the wall effect, the rate of mass transfer limitation and the enrichment of by-products had become apparent.

The experiments have shown that “reversing the direction” of the glucose supply in the aerosol culture box has been successful. An increased rate of production with an improved quality of the final product was possible.

Because of the direct supply of the glucose to the active cells, the diffusional problems, described in previous chapters no longer exist. In principle, the fermenter can proceed for time periods which far exceed what was possible previously.

In the conventional surface culture the aerobic zone does not change position and the product has to move downwards into the substrate. This movement is hindered by the wall effect. However, in the aerosol system the aerobic zone gradually formed on the top layer of older product which does not have to move as in the previous design.

The beaker experiments involve a constant combined volume of substrate and product. Some by-products are inevitably made and hence the concentration of these must increase in time in this constant volume system. In contrast, the aerosol bioreactor has an ever increasing volume of cellulose which will prevent the enrichment of these by-products. The conditions for the producing bacteria are always constant. This leads to a consistently higher quality cellulose being manufactured.

Further developments

The aerosol bioreactor can, in theory, be made as large as the designer wishes in order to make excellent quality cellulose, but the problem of contamination has to be solved. Clearly, sterilisation at the beginning of the process is not sufficient for such long production periods. The aerosol generator and the distribution box will need to be periodically disinfected (perhaps by steam) and, in addition, other elements of the reactor (which do not have the cellulose making bacteria on them) could be treated with an antimicrobial layer to a further reduction of the risk of infections (e.g. thermo-stable hybrid materials composed of glycoprotein and inorganic matrices [8]).

As with all manufacturing processes, the aerosol bioreactor will have to be economically attractive. The capital cost will be a major consideration because the operating costs will be relatively low because of the simple nature of the processing. The individual pieces of equipment will have different cost characteristics. For example, the culture box is expected to be a fairly low cost item, whereas the aerosol generator will be expensive. This might suggest having a number of culture boxes fed by one, large aerosol generator. This would give the overall system some of the characteristic of a semicontinuous process because one culture box could be emptied whilst another could be in the product growing phase.

The interesting observation that the degree of polymerisation of cellulose produced in the new aerosol bioreactor was half that of the static culture but the network of fibrils appeared more dense is to be investigated further. We plan to study if the aerosol bioreactor could give us the opportunity to affect the structural features of the cellulose systematically, e.g. by a variation of the aerosol volume and substrate concentration. In addition to the effect of the aerosol feeding on the structure of the product, it is also important to know the way in which microbial parameters such as the doubling time and the yield coefficient of the producing bacteria are themselves effected by the direct substrate feeding conditions. The results will be presented later.

5.6 Conclusion

The goal of this study has been the optimisation of production of bacterial cellulose. In chapters 2, 3 and 4 it was shown that the process is first of all limited by external factors. The new aerosol bioreactor eliminates these factors and offers the theoretical prospect of an unlimited, continuous production rate. The contamination problem still has to be solved. If this is achieved, the commercial mass production of high quality BC membranes could allow still more and new applications of this fascinating material.

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6 SUMMARY / ZUSAMMENFASSUNG

The aim of the present work was the development of a bioreactor for the optimised production of bacterial cellulose in surface culture. The starting point was the traditional surface culture method according to Schramm und Hestrin [1], which has an output of high quality bacterial cellulose, but is unsuitable for mass production due to its strongly limited product yield and the disproportional between the work-intensive preparation steps and the product formation phase.

With the help of the experimental data and mathematical simulations, it could be shown that the process is predominantly limited by external factors, which are more important than the microbial conversion of glucose to cellulose.

The inhibition effects of product removal is most important. If culture boxes with vertical walls are used for the production of bacterial cellulose in surface culture, there is, in the course of the cultivation, a restriction concerning the (essential) “sliding” of the cellulose layer to deeper zones of the nutritive medium. This so-called “wall effect“ stops the formation of the product depending on the use of vertical walled vessels at a time between the 10th and 30th day of cultivation.

Furthermore, it was observed that the traditional surface culture for the production of bacterial cellulose was inhibited to an lesser degree by substrate limitation of the product producing bacteria, caused by the increasing diffusive resistance to the transport of substrate.

Another negative influence is the enrichment of waste products in later stages of traditional surface culture.

In the practical part of this work, the development and testing of a surface culture reactor for the optimised production of bacterial cellulose took place. According to the obtained results, the development of this reactor was done to avoid the “wall effect“, diffusion-caused substrate limitations and the enrichment of waste products in the medium.

A new system was proved to be promising, in which the liquid culture medium was transformed into substrate aerosol by the help of ultrasonic vibrations. This aerosol then was applied constantly, gently and aligned to the need of the bacteria on the surface of a bacterial cellulose layer. This principle was realised in a bioreactor. The results achieved with this bioreactor proved the correctness of the earlier ideas because the cellulose producing bacteria reacted on the direct dynamic supply of substrate with a constant product formation rate over weeks of the cultivation. Moreover, the system delivers bacterial cellulose of excellent quality and can therefore be ranked as a basis for a scaling up of production rates for this fascinating material.

Forecast

The developed bioreactor delivers cellulose of a degree of quality, which is needed for medical applications or applications in cosmetics. However the bioreactor does not meet the requirements of modern fermentation systems concerning long time sterility, instrumentation and automation. Further

research therefore needs to include the further development of these matters of the bioreactor for the production of bacterial cellulose.

The developed bioreactor allows an increased product yield and the advancement of its quality. It also offers, for the first time, possibilities to actively vary key-parameters of the formation of bacterial cellulose such as

- residence time of bacteria within the aerobic bacterial cellulose matrix
- moisture within the bacterial cellulose matrix
- content of substrate within the aerobic bacterial cellulose matrix

Further work could clarify, the extent to which a variation of these parameters might have an influence on the structure of bacterial cellulose.

The implementation of the developed bioreactor is not exclusively limited to the production of bacterial cellulose. Indeed, the underlying principle can be seen as universal approach of a system for solid phase fermentation. Therefore, future research and development studies aim to develop an universal adaptive surface fermentation system on the basis of the new aerosol method.

The research goals have been successfully realised within the framework of the present study. A pilot installation of the new bioreactor was created, which can be used for the economic production of bacterial cellulose in commercial amounts.

Ziel der vorliegenden Arbeit war die Entwicklung eines Bioreaktors zur optimierten Produktion von Bakteriencellulose in Oberflächenkultur. Die Basis bildete das traditionelle Oberflächenverfahren nach Schramm und Hestrin [1], das zwar Bakteriencellulose in hoher Qualität liefert, jedoch aufgrund der eng limitierten Produktausbeute und des ungünstigen Verhältnisses zwischen den arbeitsintensiven Vorbereitungsschritten und der Produktbildungsphase für eine Massenproduktion untauglich ist.

Anhand der gewonnenen experimentellen Daten und der auf der Basis dieser Daten durchgeführten Simulationen konnte festgestellt werden, dass der Prozess in erster Linie durch externe, d.h. der mikrobiellen Umsetzung von Glucose in Cellulose übergeordnete Faktoren limitiert wird.

An erster Stelle ist der gehemmte Produktabtransport zu nennen. Werden zur Produktion von Bakteriencellulose in Oberflächenkultur Gefäße mit vertikalen Wandungen verwendet, kommt es im Laufe der Kultivierung zu einer Behinderung des bei diesem Verfahren essentiellen Abgleitens der Celluloseschicht in tiefere Zonen des Nährmediums. Der so genannte „Wandeffekt“ stoppt die Produktbildung je nach verwendetem Vertikalgefäß zwischen dem 10. und 30. Tag der Kultivierung.

Weiterhin wurde festgestellt, dass die traditionelle Oberflächenkultur zur Produktion von Bakteriencellulose in untergeordnetem Maße durch eine Substratlimitation der produktbildenden Bakterien, hervorgerufen durch die Hemmung des diffusiven Substrattransportes, beeinträchtigt wird.

Die bei der traditionellen Oberflächenkultur zwangsläufig auftretende Anreicherung von Ab- und Nebenprodukten der Bioreaktion übt ebenso einen weiteren negativen Einfluss aus.

Im praktischen Teil der vorliegenden Arbeit erfolgte die Entwicklung und verfahrenstechnische Umsetzung eines Oberflächenkulturreaktors zur optimierten Produktion von Bakteriencellulose. Den zuvor gewonnenen Erkenntnissen entsprechend, erfolgte die Entwicklung dieses Reaktors unter der Maßgabe, den „Wandeffekt“, das Auftreten von diffusionsbedingten Substratlimitationen und die Anreicherung von Abprodukten im Medium zu verhindern.

Als zielführend erwies sich ein System, bei dem die flüssige Nährlösung mit Hilfe von Ultraschallzerstäubern in ein Substrataerosol umgewandelt wird und dieses Aerosol dann gleichmäßig, schonend und auf den Bedarf der Bakterien abgestimmt auf die Oberfläche einer Bakteriencelluloseschicht aufgebracht wird. Dieses Prinzip wurde in einem Bioreaktor verwirklicht. Die mit diesem Bioreaktor erzielten Ergebnisse bestätigen die Richtigkeit der zuvor aufgestellten Thesen, denn die cellulosebildenden Bakterien reagieren auf die direkte dynamische Substratversorgung mit einer konstanten Produktbildungsgeschwindigkeit über mehrere Wochen der Kultivierung. Darüber hinaus liefert dieses System Bakteriencellulose von ausgezeichneter Qualität und kann daher als Basis für eine echte Massentechnologie zur Produktion von Bakteriencellulose angesehen werden.

Ausblick

Der funktionsfähige Bioreaktor liefert Cellulose in einer Qualität, die z.B. für medizinische Anwendungen und Anwendungen in der Kosmetik gefordert wird, er genügt dagegen nicht den Anforderungen hinsichtlich Handling, Betriebssicherheit und Prozessteuerung, die an eine moderne biotechnologische Produktionsanlage gestellt werden. In sich anschließenden Entwicklungsarbeiten

muss daher die diesbezügliche Weiterentwicklung des Bioreaktors zur Produktion von Bakteriencellulose erreicht werden.

Der entwickelte Bioreaktor ermöglicht nicht nur eine Ausbeuteerhöhung und die Verbesserung der Produktqualität, sondern er eröffnet erstmals Möglichkeiten, um wichtige Schlüsselparameter der bakteriellen Cellulosebildung wie

- Verweilzeit der Bakterien innerhalb der aeroben Bakteriencellulose -Matrix
- Wassergehalt in der Bakteriencellulose -Matrix
- Substratgehalt in der aeroben Bakteriencellulose -Matrix

aktiv zu variieren. In weiterführenden Arbeiten soll geklärt werden, inwieweit durch Variation dieser Parameter eine Einflussnahme auf die Struktur von Bakteriencellulose möglich wird.

Die Anwendung des entwickelten Bioreaktors ist nicht ausschließlich auf die Produktion von Bakteriencellulose beschränkt. Vielmehr kann das zugrunde liegende Prinzip als Ansatzpunkt für ein universell einsetzbares Festphasenfermentationssystem angesehen werden. Nachfolgende Forschungs- und Entwicklungsarbeiten sollten daher die Zielstellung verfolgen, auf der Basis des Aerosol-Verfahrens ein an vielfältige Anwendungsfälle adaptierbares Oberflächenfermentationssystem zu entwickeln.

Im Rahmen der vorliegenden Arbeit konnten die im einleitenden Konzept postulierten Hauptziele erfolgreich realisiert werden. Es wurde eine Pilotanlage geschaffen, die nach einer erforderlichen Weiterentwicklung die wirtschaftliche Produktion von Bakteriencellulose in kommerziellen Mengen ermöglicht und deshalb dazu beiträgt, bereits etablierte Anwendungen der Bakteriencellulose auszubauen und dieses Produkt noch weiteren Industriezweigen zugänglich zu machen.

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PUBLICATIONS LIST

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The results obtained during my work on the field of bacterial cellulose have been presented in lectures (L) and on posters (P) on the following national and international conferences:

[1] 11. Heiligenstädter Kolloquium, Heilbad Heiligenstadt, 2002
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[2] 12th International Conference on Flexible Automation & Intelligent Manufacturing, Dresden 2002
"Optimising the batch production of bacterial cellulose" **(P)**

[3] 15th International Congress of Chemical and Process Engineering, Praha 2002
First modelling experiments for optimising bacterial cellulose formation with new technologies **(L)**

[4] 15th International Congress of Chemical and Process Engineering, Praha 2002
A new technology for an optimised supply of emerged microbial cultures **(L)**

[5] 4. Internationales Symposium „Werkstoffe aus Nachwachsenden Rohstoffen", Erfurt, 2003
Bakteriencellulose – Herstellung, Modellierung der Bildung und Applikationen in Kosmetik und Veterinärmedizin **(P)**

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"Ein neuartiges Biosystem mit der Potenz zur Massentechnologie von Bakteriencellulose" **(L)**

[7] 16th International Congress of Chemical and Process Engineering, Praha 2004
Modelling the bacterial synthesis of cellulose **(L)**

[8] 7th World Congress of Chemical Engineering, Glasgow 2005
"Numerical Modelling of Bacterial cellulose manufacture" **(L)**

[9] 7th World Congress of Chemical Engineering, Glasgow 2005
"A novel aerosol system to improve the rate of production of bacterial cellulose" **(P)**

[10] Bioperspectives, Wiesbaden 2005
"Modelling the bacterial synthesis of cellulose" **(L)**

[11] 7th International Symposium „Alternative Cellulose", Rudolstadt, 2006
Bacterial cellulose-production and application: A patent survey **(L)**

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ERKLÄRUNG

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Morschen, Juni 2010 Michael Hornung