Nationaal Lucht- en Ruimtevaartlaboratorium
National Aerospace Laboratory NLR

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Self compensating real-time biomass sensor

D. van den Assem, R.C. van Benthem and A.A. Castelijn

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Summary
The present report describes the results of biomass measurements performed with a breadboard of a real-time dual ratio turbidity sensor, based on a concept as is developed at 3T B.V. (Enschede, The Netherlands) and accordingly tailored to use for biomass measurements at the NLR.
At first an eighteen days settlement test with a stabilized bacterium culture (*Xanthobacter Autrophicus GJ10*) has been performed with the sensor at NLR, showing a very slow settle-down of the bacteria and excellent long-term sensor stability.
Accordingly real-time measurement runs have been performed on growing *X. Autrophicus GJ10* cultures in a temperature-controlled room at BIOCLEAR B.V. premises (Groningen, The Netherlands). The sensor monitored the growth rate of the exponentially growing bacteria successfully, which is reported here. From the data a maximum specific growth rate, and also a sort of the growth acceleration rate, could be retrieved.
At the end of the growth phase, the sensor signal reduces abruptly by some percentages, followed by a constant signal. That phenomenon is attributed to the change of optical extinction properties of the different modes of existence (e.g. growth, stabilized, fixated, etc.) of the bacteria cells. Consequently, calibration of the turbidity sensor is required for the various ways of appearance when used as biomass sensor for bacteria, which appear in different modes of existence.

1. Introduction

Presently the interest in application of bacterial metabolism as part of conversion processes, e.g. medicine production, waste water purification, etc., is increasing rapidly. Also for this reason, it is subject of research for application onboard microgravity laboratories in space. Appropriate knowledge about bacterial behavior under conditions of microgravity, such as growth on a specific substrate, is required therefore.
Up to know growth experiments onboard spaceborne laboratories have been performed and planned with use of samples of an ensemble of separately growing cultures during different preset time periods. After recovery on earth the samples are analyzed. Such as the BIOKINetics project, of which one experiment set-up was flown on MIR (1996) and another is planned for the shuttle flight STS-107 (2001) (reference 1).
Introduction of automation of the complete experiment, in combination with real-time biomass measurement, during the bacterial growth process will improve such an experiment considerably with respect to monitoring and possible control.
In order to realize such an improvement, a team consisting of 3T B.V. (Enschede), Stork Product Engineering B.V. (Amsterdam) and the National Aerospace Laboratory (NLR) (Amsterdam) proposes to develop a Smart BioContainer. It will consist of a bioreactor, a liquid handling system, a biomass sensing system, and an appropriate control and data-handling electronics system. Within the scope of that development a biomass sensor for real-time measurement of bacterial growth in a liquid medium under conditions of microgravity is under development at NLR.

2. Sensor principle description

The sensor concept is based on dual turbidity measurement. It combines scattering and transmission measurement to reduce circuitry-induced instabilities. As such it is a measure for the biomass in a medium provided invariance of the optical properties and the geometry of the particles (e.g. growing cells), which produces the turbidity (reference 2).
The sensor development is derived from a turbidity sensor, developed and build by 3T B.V. The concept of the optical sensor head is depicted in figure 1.

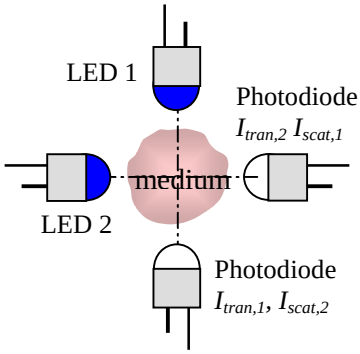


Figure-1. Dual ratio turbidity sensor concept

Two LEDs illuminate alternately the medium under investigation. Two photodiodes measure the light intensity, alternating combined transmission and scattering detection and vice versa, synchronously with the LEDs' emission. The output of the detectors is amplified logarithmically in order to increase the measurement range The signals are processed electronically to produce the logarithmic value of the geometrical mean of the alternately obtained quotients of scattered- and transmitted light as induced by each light source and detected in real-time. As a result, $\ln R^*$ is calculated on-line by:
$\ln R^* = \ln R_1 + \ln R_2 = \ln(I_{scat,1}/I_{tran,1}) + \ln(I_{scat,2}/I_{tran,2})$,
with:
R^* dual ratio signal
R_i signals ratio, induced by LED i
$I_{scat,i}$ scatterred intensity signal, induced by LED i
$I_{tran,i}$ transmitted intensity signal, induced by LED i

The ratio determination eliminates LED fluctuatons and the geometric mean calculation eliminates detector circuit instability and fouling of surfaces in the optical paths.
The detected light energy, transmitted through the medium, depends on the wavelength spectrum, the illuminated medium volume, the properties of the suspended particles (optical properties, characteristic dimensions and shape) and the suspended particle density.
As such the partical concentration (e.g. the biomass) can be measured by determination of $\ln R^*$ or the Optical Density (OD). The signal is related to the OD of the medium, as $OD = -\log(I_{tran}/I_{in})$,
with:
OD optical density value
I_{tran} transmitted intensity signal
I_{in} incident light intensity signal
The OD(450) value at λ = 450 nm, determined with a CADAS 30 spectrophotometer, is used as a measure for the biomass at the BIOCLEAR laboratory. Accordingly for the measurements with the sensor reported in the present paper the OD(450) value is used for calibration of the sensor signal, $\ln R^*$.

3. Breadboard description

The sensor performance has been verified with use of a breadboard consisting of a sensor head, made in accordance with the described concept (see fig.1), and an electronics unit for control, signal conditioning and -processing. The breadboard is made available by 3T B.V., and adapted for the present measurements.
Figure 2 presents the breadboard layout, including the peripheral equipment for operation in the laboratory.
The peripherals consist of a power supply, a data I/O interface, additional measurement equipment as required, interfaced via a General Purpose Interface Bus (GPIB) and a PC.
To suppress the influence of external light sources the sensor head, including the cuvette with liquid, is placed inside a dark cover.

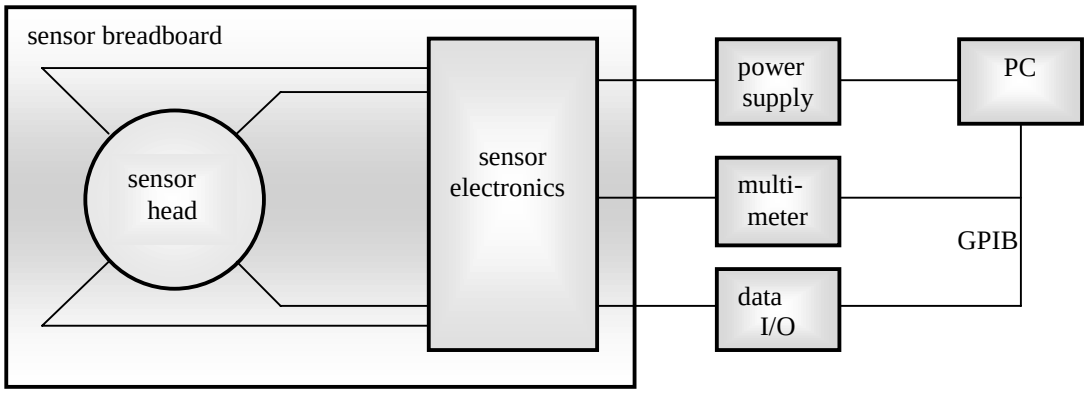


Figure-2. Breadboard layout as used for real-time measurements.

A sketch and a photograph of the sensor head breadboard are presented in figure 3 and figure 4, respectively.

The sensor head breadboard consists of a hollow, thick walled cylinder. Two LEDs and two photodiode detectors are mounted in holes in the cylindrical wall, evenly spaced at π/2 radians. Each photo-detector is

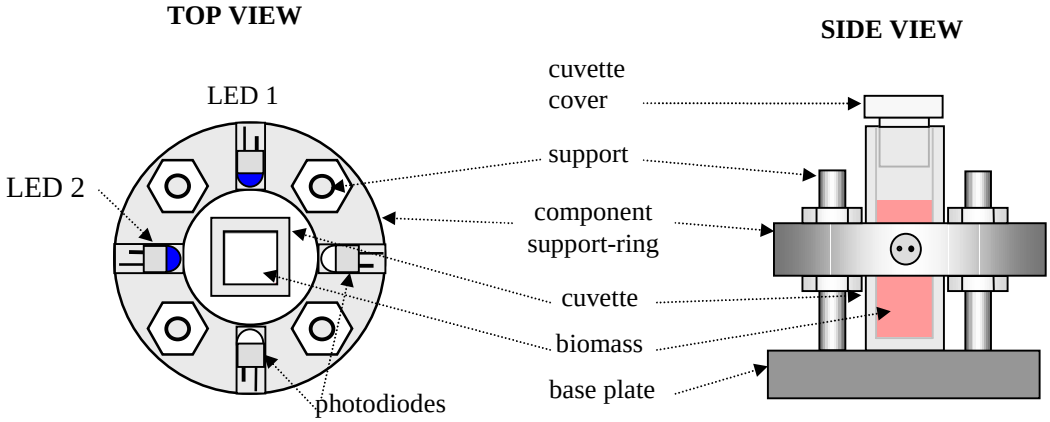


Figure-3. Optical sensor head breadboard

opposite to one LED and perpendicular to the optical axis from the other LED. A cuvette, which contains a liquid with suspended particles of which the turbidity must be determined, can be placed in the, each other crossing, optical paths between the LEDs and photodiodes.

The principle of operation of the breadboard is as follows.

A measurement run consists of a sequence of turbidity value determinations, separated by adjustably pre-set time intervals, during a long period, e.g. days.

One turbidity value is determined from the average of a number of photodetector signal readouts during the activation time period of one of the LED's.

The averaged detector circuit readouts are directly obtained from the logarithmically amplified photodetector outputs. Accordingly, the averages of the logarithmic transmitted light signal values are subtracted from the averages of the scattered light signal values.

The same procedure is followed when the second LED is switched on. The outcome of both procedures is added up, which yields the logarithm of the actual signal that represents the turbidity value.

The sensor signal is calibrated with respect to the OD or known turbidity of a reference liquid and a curve-fitting algorithm can be used to find the best analytical function match of the measured values of $\ln R^*$ with respect to the measured calibration values.

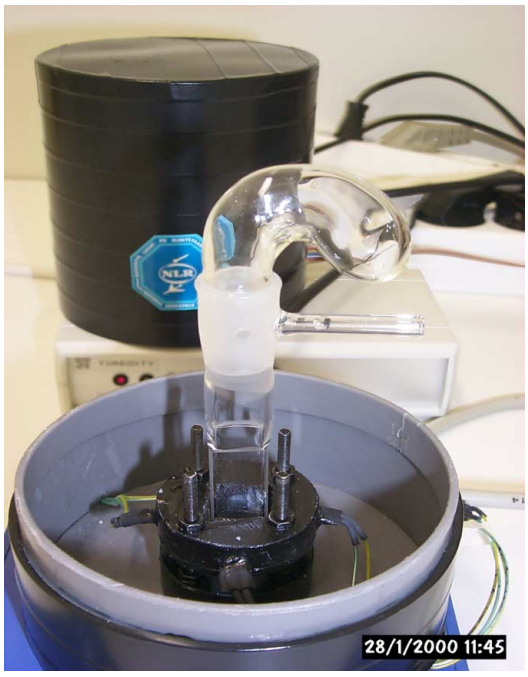


Figure-4. Optical sensor head breadboard with cuvette

Measurements for performance assessment were made with cuvettes filled with liquid samples of various concentrations of Formazin, which is usually applied as turbidity standard. Large sensitivity (better than 1 NTU (Nephelomatric Turbidity Units)) at low NTU values and a large measurement range was established. A good stability was obtained during the period that the experiments were executed. Variations <1% with a large time constant (about hours) were observed during an uninterrupted measurement period of two weeks. It is expected that these small variations are induced thermally, as no special thermal stabilization is included in the electronics. The results of the performance assessment have been reported in reference 2.

4. Settlement tests

It was observed that the fixated cells slowly sink to the bottom of their container (cuvette or flask) within a period of weeks.
Therefor a settlement test has been carried out preceding the in-situ measurements. The test has been performed with use of a cuvette with biomass consisting of fixated *X. Autrophicus GJ10* cells in the usual medium. The cuvette was placed in the sensor head.
See figure 5 that pictures the sensor head with the settlement test cuvette.
The process was monitored during a period of eighteen days, from the 16th of December 1999 until the 3rd of January 2000. During the first four days, the sampling rate was once every 5 minutes.
After four days the sensor head cover was removed a few minutes for visual inspection of the progress of the experiment. Hereafter the measurement rate was set to once every 30 minutes.
The test results are presented figure 6.
It shows the registered sensor signal (in OD values), including a (sort of) settling rate (in OD/day values). The settling rate was derived from the curve registered OD curve, calculating the time derivative of a one-hour moving average.

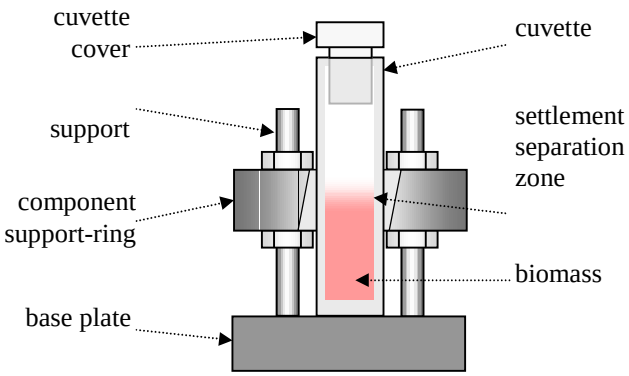


Figure-5. Settlement tests sensor head breadboard set-up

During the settling process the cells, forced by gravity, move downwards at a constant rate.
The curve starts at an OD of about 0.8 ends with an OD of 0.05. At the moment of the visual inspection and change of measurement rate (after 4 days) a sharp irregularity is observed, as is to be expected.
From the figure, and also by visual inspection, a relatively sharp separation zone was observed between the clear medium and the dispersed cells. It indicates that the difference of cell's size and weight is relatively small.
The slight asymmetry of the decreasing curve may be explained by a gradually decreasing cell concentration until the separation zone passes the detection zone.

The separation zone entered the sensor detection range after about 6 days, and left the detection range after about 12 days, leaving the clear medium liquid behind. The detection height range was approximately 10 mm, hence the settling rate is calculated roughly to be 1.6 mm/day.
The sensor showed good stability and sensitivity during the eighteen days period of observation, apart from a small anomaly at the seventh day and a small ripple. The latter, most possibly, indicates that the sensor electronics is slightly sensitive to temperature variations. Introduction of means for temperature stabilization will cope with the problem, when required.
Additional scattering might cause the small anomaly when the separation zone starts entering the detection zone.

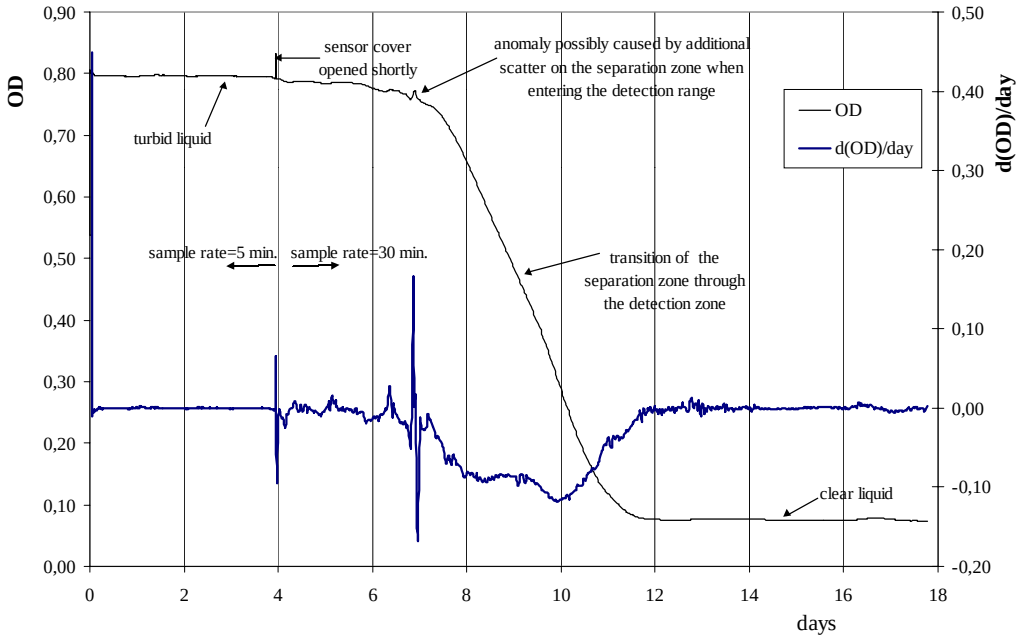


Figure-6. Presentation of the OD value obtained from the real-time measured sensor signal (OD calibrated verses lnR) when the separation zone passes the detection zone of the sensor, with the calculated OD rate (OD/day) included.*

5. Calibration

The dual ratio turbidity readings of the sensor breadboard were calibrated with respect to OD readings of the CADAS 30 photospectrometer, available at the BIOCLEAR laboratory.
The calibration has been performed with eight cuvettes one of which contains medium only and seven were filed with medium with different concentrations of fixated *X. Autrophicus GJ10* cells. See figure 7.
Of each of these cuvettes, the response signal $\ln R^*$ of the biomass sensor, and the photometric OD value at $\lambda = 450$ nm has been measured. Accordingly a plot has been made of the OD values against the sensor output signal, $\ln R^*$. Therefrom a calibration curve was obtained with use of curve fitting. See figure 8.

*Figure-7. Overview of the measurement cuvettes.
One measurement cuvette with air reservoir.
Eight measurement cuvettes, filled with liquid medium and fixated bacteria of various concentrations for calibration purposes.*

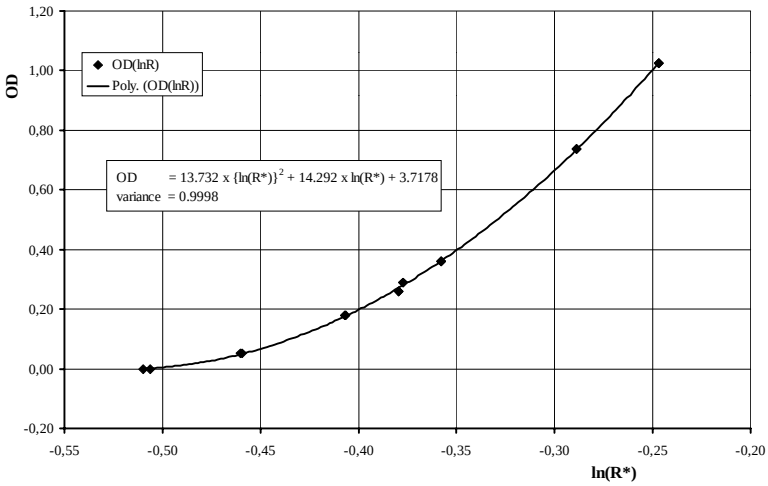


Figure-8. Calibration curve.

6. Biomass measurements

The in-situ performance of the sensor as biomass sensor has been verified by observation of the bacterial growth of a *X. Autrophicus GJ10* in a liquid medium with alcohol as a nutrient. The measurements with the sensor breadboard were accompanied by reference measurements.

During the measurements it turned out that the medium with the growing bacteria has to be stirred. Within the limits of the used equipment the stirring rate turned out to have no influence on the biomass determination

The measurements have been performed in a temperature-controlled room at the BIOCLEAR premises. BIOCLEAR supported the experiments and prepared the bacteria culture.

See figure 9 for the set-up in the temperature controlled room.

Figure 10 presents the growth curve, composed from the turbidity sensor data, with use of the calibration data obtained as described in the previous section.

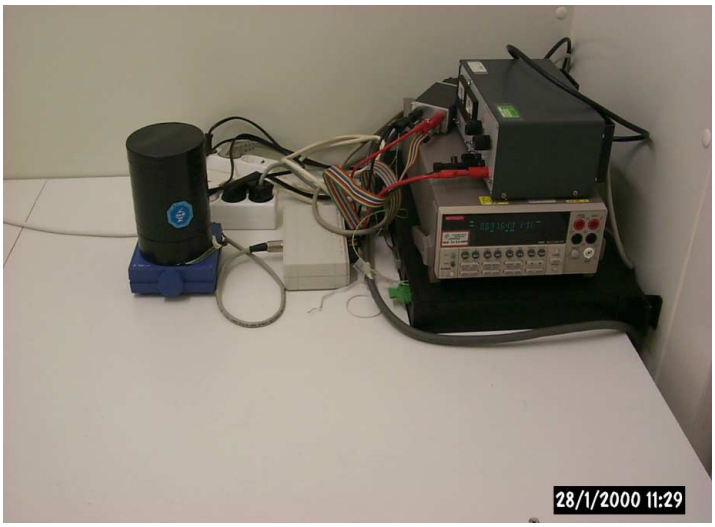


Figure-9. The biomass sensor breadboard set-up on a table in the temperature-controlled room at BIOCLEAR. Note the magnetic stirring device on which the sensor head is placed.

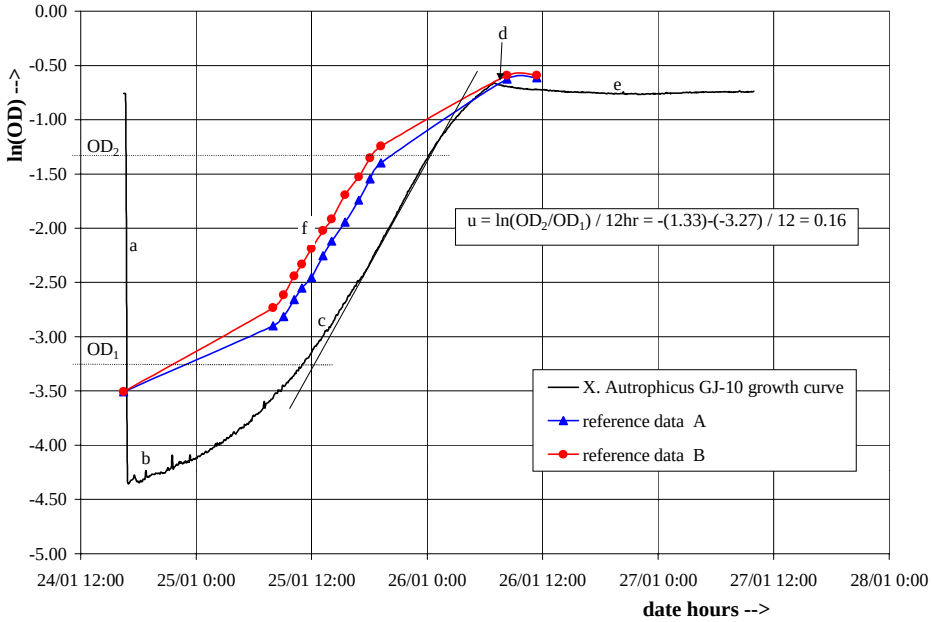


Figure-10. X. Autrophicus GJ-10 growth curve, with geometrical estimation of the maximum growth rate (u). Two sets of reference measurement data are included. The figure shows:
the switch-on of the instrument (a),
the lag-phase (b),
the phase of exponential growth (c),
the complete consumption of substrate (d),
the stationary phase (e) and
the sample measurements from two separately growing reference cultures (f).

At the termination of the growth, and the on-set of the stationary phase of the measurement run, the signal curve changes abruptly from an increasing- to a decreasing course, as can been seen in figure 10, point (d). A probable explanation might be that active growing bacteria cells show different scattering properties with respect to those in the stationary phase, when they are out of nutricients, required for growth.
The logarithm of the measurement curve can be used to estimate maximum growth rate of the bacteria. Assume an exponential growth of the number of cells N_t or OD: $N_t = N_o^{\mu(t)*t}$. With N_t being the number of cells at time t, N_0 the number of cells at t=0 and $\mu(t)$ the specific growth rate Then the time derivatives of $\ln(N_t) = \mu(t)t + C$ give the specific growth rate $d\ln N_t/dt = \mu(t)$ as function of the time.
The maximum specific growth rate can be derived directly from the maximum slope of the ln(OD) curve giving: $\mu_{max} \approx 0.16$, which shows to be comparable with those, obtained from the reference curves. In this case the maximum specific growth rate of the sensor measurements, can be expressed in "ln(OD) per hour units".
A different approach of maximum growth rate estimation consists of calculation of the first derivative from the

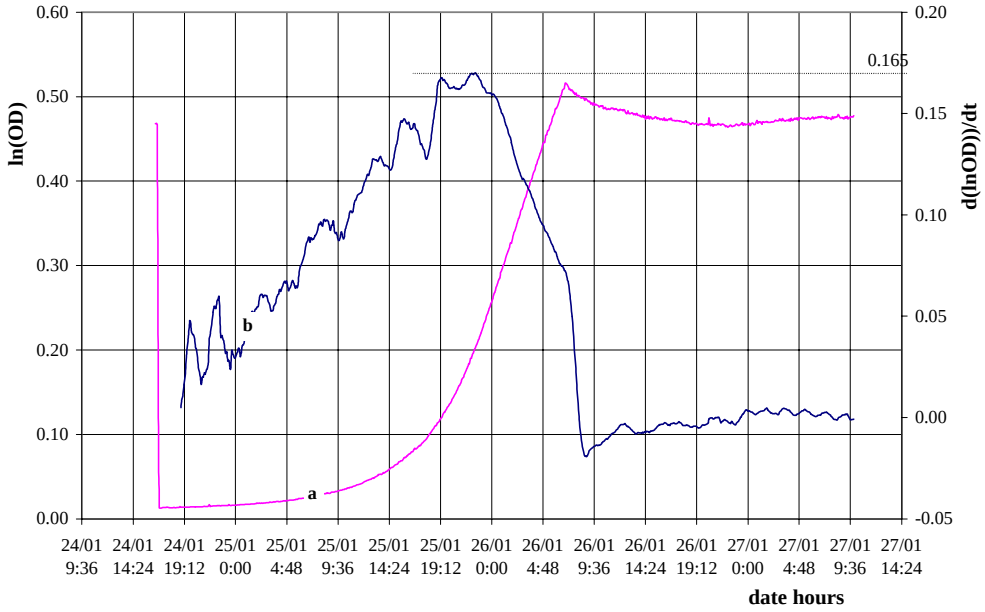


Figure-11. Results of the second real-time measurement run
a. The measured OD growth curve.
b. The calculated one hour moving average of the growth rate (d(lnOD)/dt)

growth curve of figure-10. Figure-11 presents a one hourly progressive average growth rate (d(lnOD)/dt) calculated as function of time from the curve of figure 10. This growth rate increases fluctuatingly until the maximum of $\mu_{max} \approx 0.165$, measured in units ln(OD)/hr, is attained. As expected the value corresponds sufficiently with the one obtained geometrically.

7. Conclusions

The turbidity sensor breadboard shows an excellent long-term stability as can be concluded from the settlement test. The remaining small fluctuations are attributed to temperature in the electronic circuitry. These can be stabilized if required.
The sensor can be calibrated with use of a set of media with different concentrations of bacteria cells, provided that the optical extinction properties of the cells do not change during the time period between calibration. The observed calibration reproducibility is about OD $\approx \pm$ 0.01. However, most possibly a different set of calibration data must be determined for each mode of existence of the cells.
The value of the maximum growth rate can be derived from the course of the growth curve, either geometrically, or by calculation.

The growth phase ends abruptly, decreasing slowly by a few percentages to a stable phase. A change of optical extinction properties of the bacteria cells, when turning from a growing state into an inactive state of existence may explain this phenomena.
During the course at the measurements it turned out that stirring of the medium is required. Within the limits of the used magnetic stirring device, the measurements were not measurably influenced by the stirring rate.

References

1. Waarde, J.J. van der; Keuning, S.; Paul, p.g.; bonot, R.G., *Determination of the space influence on the kinetics for biodegradation of organic volatile contaminants. Proceeding of the 6th European symposium on Space Environmental Control Systems*. Noordwijk, 20-22 May, 1997.
2. D. van den Assem; *Development of a biomass sensor. Dual ratio versus transmission trade-off.* NLR-TR-99444

Acknowledgement

We owe much gratitude to 3T B.V. for providing their original turbidity sensor with electronics breadboard for adaptation, and to BIOCLEAR B.V. for the support to do experiments in their facilities at their site with the *X. Autotrophycus GJ10* bacteria culture, which they provided. We highly appreciate the stimulating discussions with Mr. Eckhard (Stork Product Engineering B.V.), Mr. Leeuwis and Mr. Prak (3T B.V.), and Mrs. Hammenga, Mrs. Krooneman and Mr. van der Waarde (BIOCLEAR B.V.).

Appendix A Real-time turbidity sensor for biomass measurement

REAL-TIME TURBIDITY SENSOR FOR BIOMASS MEASUREMENTS

Capabilities

- monitoring turbidity related liquid properties (such as biomass),
- during a long period of time,
- without the requirement of sensor head cleaning and
- without the need of regular calibration.

Sensor features

- real-time data acquisition
- on-line data processing
- a large measurement range
- a large sensitivity
- a long-term stability

Background

Stork Product Engineering B.V. (SPE) introduced the idea to use a turbidity sensor for biomass measurements, as part of a Smart BioContainer (SBC). The sensor is derived from a turbidity sensor developed and built by 3T B.V. It is being adapted for bacterial growth measurement by NLR. The biomass sensor is currently being developed aiming for the BIOKIN-3 ground reference experiment.

Sensor concept

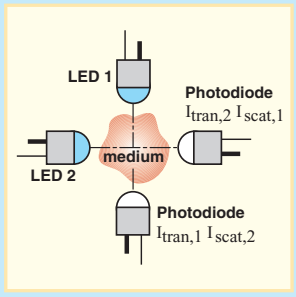
The sensor concept is based on dual turbidity measurement. It combines two scattering and transmission measurements.
The output of the detectors is amplified and on-line processed electronically in order to produce the geometrical mean value of the two alternately obtained quotients of scattered and transmitted light:

$$R^* = R_1 \times R_2 = (I_{scat,1} / I_{tran,1}) \times (I_{scat,2} / I_{tran,2})$$

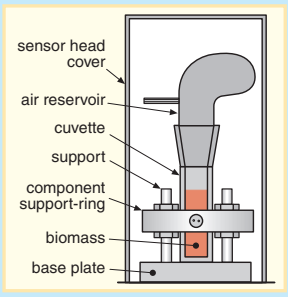
The ratio determination eliminates LED emission fluctuatons.
The geometric mean calculation eliminates detector circuit instability and fouling of surfaces in the optical paths.
R* measures the biomass in a medium unattended during long time periods.
Optical properties and geometry of the particles (e.g. growing cells) which produce the turbidity requires appropriate calibration.

Breadboard description

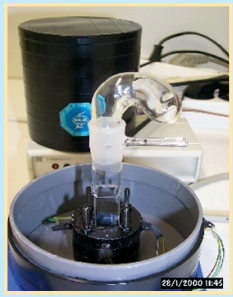
The sensor performance has been verified with use of a breadboard. The breadboard includes the sensor head and an electronics unit. Two LEDs and two photodiodes are mounted in a support ring. A cuvette with air reservoir, which contains the biomass liquid, is placed in the support ring. A cover is placed over the sensor head for rejection of light from outside.



Dual ratio turbidity sensor concept



Optical sensor head breadboard



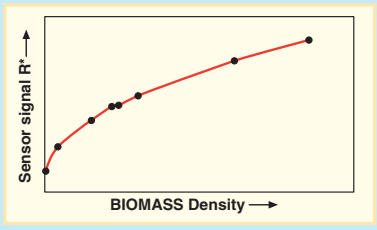
Optical sensor head breadboard with cuvette in measurement position

Calibration

The calibration has been performed with eight cuvettes which contain a medium with fixated *Xanthobacter Autrophicus GJ10* cells of different concentrations.
In order to compensate for temperature and possible other external influences, the sensor set-up has been placed inside a temperature-controlled room at BIOCLEAR premises.

Of each of these cuvettes, the response signal R* of the biomass sensor, and the biomass density at λ = 450 nm has been measured.
Accordingly a plot has been made of the sensor output signal, R*, against the OD values. Therefrom a calibration curve was obtained with use of curve fitting.

Overview of the measurement cuvettes used. Eight measurement cuvettes, filled with liquid medium and fixated bacteria at increasing concentrations for calibration purposes. One measurement cuvette with air reservoir for the growth measurement



Calibration curve

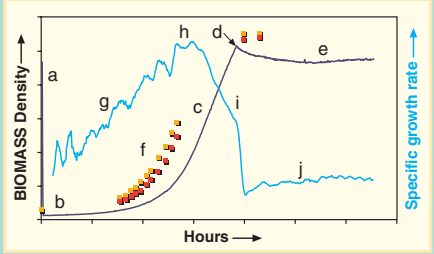
Real-time Biomass Measurements

With the described breadboard, the growth of the *Xanthobacter Autotrophicus GJ10* bacteria population in liquid medium has been recorded during a few days, with a five minutes sample rate.
The figure shows:

- The growth curve typical for *Xanthobacter Autotrophicus GJ10*, which indicates:
  - *(a) the switch-on of the instrument*
  - *(b) the lag-phase*
  - *(c) the phase of exponential growth*
  - *(d) the complete consumption of substrate, and*
  - *(e) the stationary phase*
- Sample measurements from separately growing reference cultures:
  - *(f) two sets*
- The time derivative curve, which indicates:
  - *(g) growth acceleration*
  - *(h) maximum specific growth rate, μ_{max}*
  - *(i) growth deceleration*
  - *(j) stabilisation*

Future applications

- glass sensor integrated with bioreactor
- submerged probes
- wireless transponder sensor submerged in liquid
- oil fouling monitoring (aircraft)
- spectral turbidity measurements
- milk quality monitoring (milking robots)
- Smart BioContainer (part of)



Bacterial growth, specific growth rate and reference measurements

Examples of future application. Submerged probe and glass sensor integrated with bioreactor

Acknowledgement

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