Laboratory Exercise

Teaching Bioprocess Engineering to Undergraduates: Multidisciplinary Hands-on Training in a One-Week Practical Course[S]

Marius Henkel†*
Michaela Zwick‡
Janina Beuker†
Judit Willenbacher‡
Sandra Baumann‡
Florian Oswald‡
Anke Neumann‡
Martin Siemann-
  Herzberg§
Christoph Syldatk‡
Rudolf Hausmann†

From the †Institute of Food Science and Biotechnology, Department of Bioprocess Engineering, University of Hohenheim, 70599 Stuttgart, Germany, ‡Institute of Process Engineering in Life Sciences, Section II: Technical Biology, Karlsruhe Institute of Technology (KIT), 76131 Karlsruhe, Germany, §Institute of Biochemical Engineering, University of Stuttgart, 70569 Stuttgart, Germany

Abstract

Bioprocess engineering is a highly interdisciplinary field of study which is strongly benefited by practical courses where students can actively experience the interconnection between biology, engineering, and physical sciences. This work describes a lab course developed for 2nd year undergraduate students of bioprocess engineering and related disciplines, where students are challenged with a real-life bioprocess-engineering application, the production of recombinant protein in a fed-batch process. The lab course was designed to introduce students to the subject of operating and supervising an experiment in a bioreactor, along with the analysis of collected data and a final critical evaluation of the experiment. To provide visual feedback of the experimental outcome, the organism used during class was Escherichia coli which carried a plasmid to recombinantly produce enhanced green fluorescent protein (eGFP) upon induction. This can easily be visualized in both the bioreactor and samples by using ultraviolet light. The lab course is performed with bioreactors of the simplest design, and is therefore highly flexible, robust and easy to reproduce. As part of this work the implementation and framework, the results, the evaluation and assessment of student learning combined with opinion surveys are presented, which provides a basis for instructors intending to implement a similar lab course at their respective institution. © 2015 The Authors Biochemistry and Molecular Biology Education published by Wiley Periodicals, Inc. on behalf of International Union of Biochemistry and Molecular Biology, 43(3):189–202, 2015.

Introduction

Bioprocess engineering is a highly interdisciplinary field of study, and as such, demands for practical courses where students can actively experience the interconnection between biology, engineering, and physical sciences. This, in addition to appropriate teaching methods, requires a carefully shaped program to ensure that students are not overwhelmed by the complex content of the course. These issues have been addressed in the practical course "Bioprocess Engineering" at the Karlsruhe Institute of Technology (KIT, Karlsruhe, Germany). The course is the product of a very thorough long-term development process: a comprehensive 1-week practical course which combines elements from different areas of expertise in a multidisciplinary environment. A practical

laboratory course comprising a high cell density fermentation of *Escherichia coli* strain BW3110 was initially developed by Dr. Martin Siemann-Herzberg and Prof. Dr. Matthias Reuss over a decade ago at the Institute of Biochemical Engineering (University of Stuttgart, Germany). Since that time, they fostered its development in a complex process, implementing improvements both from a scientific as well as from a didactic point of view. Nowadays, that course is still part of the curriculum at the University of Stuttgart, and held on a regular basis for students in biotechnology, bioprocess engineering, and related disciplines on an industry-related scale. The general concept of the experiment performed in that course was adopted, modified and improved to be performed on a smaller laboratory-scale, and introduced at the KIT for the first time in 2009.

The experiment performed during the practical course is focused on the production of a recombinant protein in *Escherichia coli* upon induction and application of specific feeding strategies. The entire experiment, starting from the preparation of the seed culture, the setup and installation of the bioreactor, the conduct and control of the experiment, and sampling through the analysis of the online and offline process data to a final critical evaluation of the experiment are performed by the students. Enhanced green fluorescent protein (eGFP) was chosen as the recombinant protein to be produced, since this allows for direct and easy qualitative visualization of the product by subjecting the broth to ultraviolet light. A 2.5 L small-scale fermentation platform with online measurement of pH and pO_2 as well as exhaust gas evaluation (x_{O2} and x_{CO2}) was used. Additionally, a digital peristaltic pump and laboratory scale were connected to a computer to control the flow of feeding solution in fed-batch mode. Even though this setup provides an extensive view on the principles of bioprocess engineering, the lab course can essentially be performed with bioreactors of the simplest possible design, or even retrofitted with additional equipment.

In general, literature on experiments in bioreactors designed and evaluated specifically for application in teaching is scarce. Therefore, the purpose of this work is to provide a basis for instructors who plan on implementing or reworking a lab course for biotechnology at their respective institution. In addition to the experimental setup and required protocols, the main concept behind this teaching approach, assessment of student learning as well as data on evaluation of the lab course is presented. As part of the curriculum at the KIT, the lab course is subject to an ongoing official evaluation of teaching quality. The evaluation is performed within the frame of a program established at all universities of the German state of Baden-Wuerttemberg, and the results from monitoring and evaluation from classes of 2012 to 2014 are presented in this work. In addition, the lab course received the award for excellence in teaching of the KIT faculty of Chemical and Process Engineering in the year 2014. The lab course as presented in this study is not limited or restricted to the exact experimental setup, providing flexibility for implementation elsewhere. From the experience that could be acquired during the last years and the careful assessments performed, this study can be beneficial for instructors in biology, bioprocess engineering or related disciplines.

Intended Audience, Timeframe, and Prerequisite Student Knowledge

The laboratory exercise is aimed mainly at undergraduate students in bioprocess engineering and related disciplines, and it is part of the curriculum at the end of the second year of studies. The course is part of the lecture "Bioprocess Engineering" held at the KIT, where students learn about the basics of bioprocess engineering in practice. In addition, students who are aiming for a bachelors or a masters degree in related disciplines may chose to attend the course, either as an elective subject or for non-credit advanced training. In general, most of the students from other disciplines major in chemical engineering or biology. Consequently, the broad spectrum of participants from different disciplines asks for carefully designed teaching approaches with respect to the individual degrees of knowledge in the covered disciplines.

The class is designed as a one-week full-time course with approximately 50% in-lab time and 50% out-of-lab time for small lectures and tutoring, analysis, and interpretation of data, as well as preparation of a lab journal. In its current setup, the course can be completed in one week, however, time for preparation may be saved, e.g. if student instructions are provided or required solutions are prepared by assistants/instructors in advance. Due to the nature of an experiment in a bioreactor, it is difficult to spread over several weeks, and one full week should ideally be reserved for the course. However, there are several other ways the laboratory exercise can be modified to be suitable for an individual curriculum, which are discussed in section Possible Modifications.

Students should ideally be familiar with basics in mathematics and physics, chemistry, mechanical sciences, and engineering as well as biology (e.g. pH, mass transfer, exponential growth...). Since the experiment is designed for students from different fields of studies, the instructions are given and the experiments are supervised in a way that only very little laboratory skills are required in advance. Even though previous knowledge and experience in bioprocess engineering may be beneficial, the course can easily be completed by biology majors, who in general did not attend a bioprocess engineering lecture prior to this lab course.

Learning Objectives and Teaching Concepts

The main teaching concept which is pursued during the entire lab course is the involvement and participation of the students in all activities related to the experiment. Students are challenged with a real-life bioprocess-engineering application, the production of a recombinant protein in a fed-batch process.

'Tell me, and I will forget. Show me, and I may remember. Involve me, and I will understand'
(Confucius, around 450 BC)

During the practical course students can actively experience the interconnection between biology, engineering, and physical sciences. Each group of students is assigned to a bioreactor and put in charge of their own experiment, including the preparation of the culture medium, the seed culture, the setup and calibration of the equipment, and the sampling procedure up to a critical evaluation of the data. This way, students are provided with hands-on experience of application oriented biotechnology by conducting an experiment in a bioreactor for the first time. During the lab course, students are provided with a task, which has real-world application, since many industrial applications rely on similar processes and equipment (e.g. for the production of recombinant protein in the pharmaceutical sector), although on a larger scale. The visual feedback is a vital component of the experimental outcome, the experiment, from a scientific point of view, has been designed to be highly robust in terms of achieving the desired outcome of eGFP production.

The development of learning objectives was supported by "constructive alignment" [1], an approach which combines outcome-based teaching and a constructivist presentation of the learning material. The main objective of this approach is that students are able to construct meaning from what they learn. This method is highly suitable for a practical course, since students are able to apply what they learned in preunit lectures and briefings in practice during the lab course. Furthermore, these methods favor autonomous learning of the students, and were therefore deemed suitable especially to represent learning objectives of a practical course. The learning objectives themselves were verbalized and classified by referring to "Blooms Taxonomy" [2]. The alignment of learning goals, activities and methods for assessment of student learning is presented in Table 1. Since the desired learning outcome should provide the students with theoretical, practical as well as writing skills, different methods for their assessment are required, as described in Table 1.

The basic concept behind real-world tasks in teaching applications is thought to enhance students understanding of the subject matter, as well as to benefit the development of critical-thinking skills [3]. A scientific teaching approach [4] for experimental research was used to enhance students engagement in learning by inclusion of several activities during lectures and tutorials, which is summarized in Fig. 1. As part of the scientific teaching approach, these methods have previously been reported to improve learning outcome for the students [4]. Several active exercises are performed in small groups (e.g. the development and organization of an experimental schedule), to further benefit students teamwork abilities. However, the complexity of the multidisciplinary theoretical knowledge demands for other methods of teaching besides an instructed do-it-yourself approach. It is for this reason that the lab course has been split into two basic learning units: (1) teaching of the theoretical background and workflow in active lectures, which are built around an active learning process with tutorials to involve the students, and (2) a supervised do-it-yourself unit, which allows the students to apply the theoretical knowledge, thereby strengthening their understanding of the complex workflows. During the progress of the lab course, these two basic units are alternating. Combined with special group exercises during the lectures, this concept is meant to keep students active during the teaching of theoretical knowledge.

An overview of the general concept and the schedule, along with teaching methods is given in Fig. 1. The first day of the lab course starts with a lecture which gives an introduction to the topic and outlines principles and tasks for the students in the next days. During the lecture, basic knowledge and relevant parameters are collected by the students, which are required to perform the experiment. This is implemented as teamwork exercises and group discussions (e.g. think-pair-share), which are placed around the theoretical lecturing to keep the students active at all time. Each of the following 3 days starts with a tutorial, to discuss and brief the students on what specific steps are necessary on each individual day. Several teaching methods are applied to involve the students directly in the entire sequence of operations. Directly after the tutorials, students actively experience what they learned from the lectures in the lab. This makes the lab course highly suitable for interdisciplinary participants, since many aspects of biotechnology are covered in both theory and practice. In addition, when students are assigned to the bioreactors, groups from different disciplines are favored (e.g. biology and bioprocess engineering majors), which is intended to further enhance the students cross-discipline communication skills.

Scientific Background
Biological and Genetic Background
Green fluorescent protein (GFP) was originally isolated from the jellyfish *Aequorea victoria* [5]. It consists of 238 amino acids with a molecular weight of 26.9 kDa. A similar protein can also been found in the sea pansy *Renilla reniformis* [6]. Upon exitation of GFP with ultraviolet light (maximum at 395 nm), an emission of green light (maximum around 509 nm, depending on the protein) can be detected. The gene for GFP has been isolated and become a very useful tool in certain experiments, e.g. as a nontoxic fluorescent marker or as means to study protein-protein interactions. Enhanced green fluorescent protein (eGFP) is a modified version of GFP, with higher fluorescence intensity and longer stability [7], which is well-established in the scientific community. The heterologous expression of the product enhanced green fluorescent protein (eGFP) is performed in *E. coli* strain BW3110, a rhamnose negative ($\Delta rhaB$) mutant of W3110 [8]. For expression

TABLE 1	Alignment of learning objectives (LO), activities and methods for the assessment of student learning

Learning objective (LO)	Activities	Assessment
LO 1 Students will be able to plan and execute experimental procedures. They understand how fermentation data are collected.	development of experimental schemes, sampling and sample processing	Interview (Supporting Information S4)
LO 2 The students are able to document and analyse the data in a correct and scientific way.	documentation and interpretation of the collected data in a lab journal	Lab journal (Supporting Information S4)
LO 3 Students understand the basic functions of a bioreactor and apply this knowledge during the lab course	sketching the setup and construction of the bioreactor, calibration and initialization of sensors (e.g. pH)	Short written exam, questions in category A (Supporting Information S2)
LO 4 Students will be able to analyse and interpret key elements of the fermentation data to operate the bioreactor accordingly.	exhaust gas analysis (e.g. RQ), calculation of oxygen transfer (e.g. OTR), amount of substrate left	Short written exam, questions in category B (Supporting Information S2)
LO 5 Students understand the principles of the inducible genetic expression system.	induction of protein production with rhamnose, start and control of feeding	Short written exam, questions in category C (Supporting Information S2)
LO 6 Students understand the principles of fed-batch experiments in *E. coli*, and how catabolic repression affects protein and biomass yield.	setup of the feed solution, calculation of yields, quantification of remaining glucose and acetate produced	Short written exam, questions in category D (Supporting Information S2)

Assessment data from classes of 2013 and 2014 (total of 121 students) for LO 1 and 2 by interviews and written lab journals are shown in Table 4. Further details on the assessment are available as Supporting Information (S4). Written exams were used to assess student learning regarding LO 3-6 by comparing prelab and postlab data (Table 3). Prelab written exams were performed anonymously by randomly generated tests containing at least 2 questions from each category. Post-lab written exams were structured similarly and each category was evaluated separately. Sample questions for each category are available as Supporting Information (S2).

of eGFP, a plasmid construct (pJOE4056.2) which contains eGFP under control of the rhamnose inducible promoter *rhaBAD* [9] has been used. A process for high-cell density fed-batch cultivation for this expression system has been developed and optimized [10], which served as a basis for the course described in this study. The promoter itself is furthermore catabolite-repressed, which, besides induction with rhamnose, requires for controlled levels of glucose to induce protein expression. Only under conditions of glucose limitation, enough cAMP is present to interact with cAMP

TABLE 2	Fermentation parameters in Batch, Fed-Batch 1 and Fed-Batch 2 mode; representative values and range of parameters in brackets of 28 experiments (class of 2013)

	BDM_{max} [g]	μ_{max} [1/h]	$Y_{X/S}$ [g/g]	OTR_{max} [mgO_2/s·l]	k_La [1/h]	$Y_{X/O2}$ [g/g]
Batch	1.5 (0.9–2.2)	0.54 (0.35–0.65)	0.50 (0.30–0.65)	0.20 (0.10–0.35)	0.04 (0.04–0.18)	0.72 (0.40–0.80)
FB 1	3.39 (1.3–3.5)	0.21 (0.10–0.40)	0.59(0.15–0.60)	–	–	–
FB 2	17.77(7.0–18.0)	0.08 (0.05–0.20)	0.38 (0.10–0.60)	–	–	–

FB: fed-batch, BDM: biodrymass, μ_{max}: maximum specific growth rate, $Y_{X|S}$: substrate to biomass yield (here: glucose to biomass), OTR: oxygen transfer rate, k_La: volumetric oxygen mass transfer coefficient, $Y_{X|O2}$: oxygen to biomass yield.

receptor protein (CRP), which then undergoes conformational changes to act as a transcription factor thus enhancing expression of the eGFP gene [11].

Strategies for appropriate feeding were developed according to studies, which focused on investigation of regulatory mechanisms under carbon-limitation in *E. coli* [12] and metabolic flux analysis in carbon limited fed-batch processes [13]. A sustained induction upon addition of rhamnose is accomplished, since the strain used in the experiment is unable to metabolize the added rhamnose. To reduce the amount of misfolded protein aggregations in inclusion bodies, the temperature is lowered to 30°C in the second fed-batch.

General Background for Fed-Batch Cultivations

The experiment is divided into three parts. First, a sufficient amount of biomass is produced in a batch process. Second, exponential substrate feeding begins in a fed-batch process with a high growth rate to yield a high cell density. Third, growth rate is lowered by reducing the flow rate of the feeding solution. Since the overall process time is around 24 hours depending on the setup, the second fed-batch is usually performed overnight. During the fed-batch processes, the flow of glucose has to be tightly controlled. If the concentration of glucose in the culture broth exceeds a certain level (above approximately 10–50 mg/L, depending on the strain), the cells exhibit overflow metabolism and acetate is accumulated. If acetate, which is toxic to the cells and interferes with product formation, is allowed to form, the growth rate of the cells decreases, thereby consuming less glucose, which will consequently lead to an accumulation of glucose and an even higher rate of acetate production. These facts may ultimately result in cell death and a failed experiment with low levels or absence of eGFP.

To circumvent this problem, after all the glucose has been consumed in the batch phase, the cells are allowed to metabolize the acetate in the culture broth. This process usually takes approximately 45 minutes. After that, in the following two fed-batch processes, the required amount of glucose is calculated for the desired growth rate and the feed is set up accordingly. This way, it can be assured that glucose accumulation during biomass production and expression of the eGFP protein is minimal. It is therefore evident that such high biomass yields along with eGFP production can only be achieved by using a tightly controlled fed-batch process.

Conducting and Monitoring the Experiment

During the time of the experiment, the students are supervising and monitoring the process, to determine the point when all glucose has been consumed. The enzymatic offline-determination of glucose levels in the culture is one option to achieve this, however, this usually results in long waiting periods which strongly influences the process if cells are left in a state of starvation for some time. The
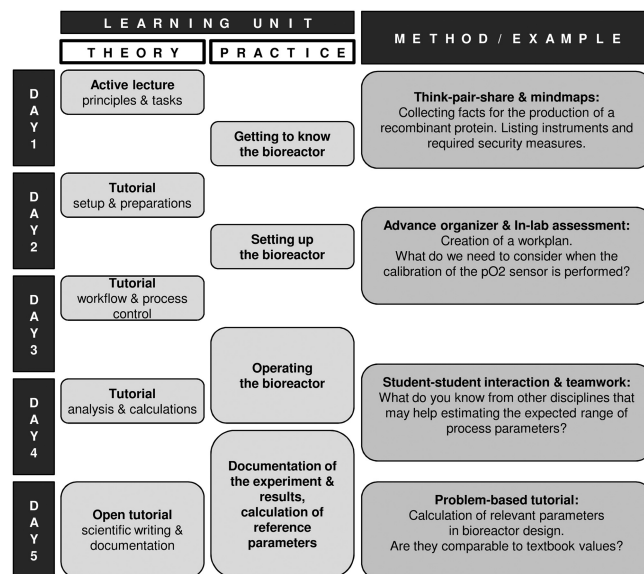

FIG 1 Overview of the general schedule, teaching units, and appropriate teaching methods.

experimental setup and hardware provides the operator with a couple of methods to determine when glucose has been depleted. To determine the growth state of the cells, online data on pO_2, exhaust gas analysis and acid/base addition may be used. Once the glucose in the batch phase is depleted, the amount of oxygen required for growth is immediately reduced. If the stirring and gassing rate are kept constant, this results in a sudden rise of dissolved oxygen in the culture broth, revealed by a rise in pO_2. Once the cells adjusted to acetate as a new source of carbon, the pO_2 starts to decline again slowly, indicating cell growth. Once the acetate is consumed, a second increase in pO_2 occurs. This indicates the point where the feeding should begin. A sudden rise of pO_2 in this case is also accompanied by a rising oxygen content in the exhaust gas, along with decreased production of carbon dioxide. The consumption of the individual substrates and growth of the bacteria may also be monitored by pH titration. During growth on glucose, the nitrogen source is simultaneously consumed. Therefore, due to the removal of ammonia from the medium, base is added to the bioreactor to maintain a constant pH. Once glucose is consumed and the metabolization of acetate begins, the addition of base subsides and acid is required to account for a rising pH due to decreasing amounts of acetate.

Experimental Procedure and Setup

A brief description of the general experimental setup and procedure is provided in the following section. A more detailed description of required chemicals and solutions as well as student instructions and templates/worksheets is available as Supporting Information (S1, S3, and S4).
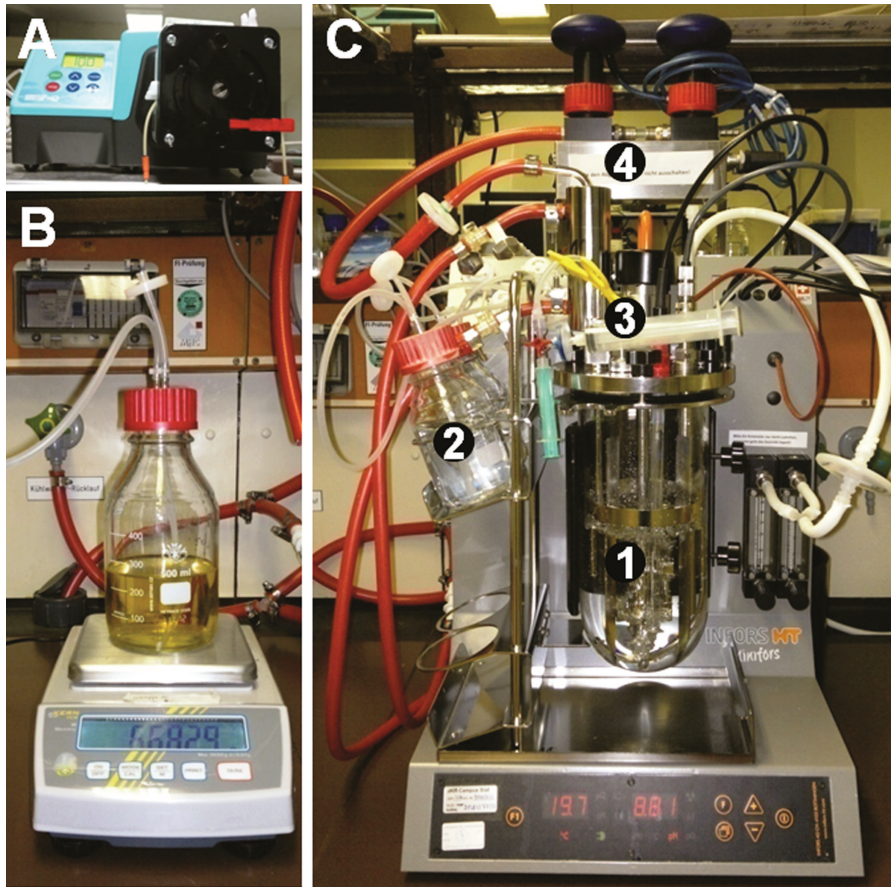
FIG 2 *The bioreactor and peripherals used during the lab course. (a) Peristaltic precision feed-pump. (b) Bottle with feed solution on a scale. (c) Bioreactor with 1. glass vessel and stirrer, 2. acid/base feeding, 3. sampling valve, 4. exhaust gas analyzer.*

Materials and Methods

Chemicals

Spectroquant® ammonia test was obtained from Merck KGaA (Darmstadt, Germany). Glucose enzymatic assay and acetate enzymatic assay was obtained from R-Biopharm AG (Darmstadt, Germany). Yeast extract and tryptone were from BD Biosciences (Heidelberg, Germany). Other chemicals were from Carl Roth GmbH (Karlsruhe, Germany). All chemicals were of analytical grade.

Media

The initial preculture was grown in lysogeny broth (LB) which contained 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g L^{-1} sodium chloride [14]. The seed culture was cultivated in mineral salt medium with 5.0 g L^{-1} NH_4Cl, 14.6 g L^{-1} K_2HPO_4, 3.6 g L^{-1} $Na_2HPO_4 \times 2$ H_2O, 5.6 g L^{-1} Na_2SO_4, 0.27 g L^{-1} $MgCl_2 \times 6$ H_2O complexed with 1 g L^{-1} sodium citrate $\times 2$ H_2O, 0.13 g L^{-1} $CaCl_2 \times 2$ H_2O, 0.01 g L^{-1} thiamin, and 10 g L^{-1} glucose. A total of 3 mL L^{-1} trace element solution and 100 mg L^{-1} ampicillin were added. The pH-value was adjusted to 7.0. The trace ele-

ment solution contained 25.8 g L^{-1} sodium citrate $\times$ H_2O, 0.18 g L^{-1} $ZnSO_4 \times 7$ H_2O, 0.10 g L^{-1} $MnSO_4 \times H_2O$, 8.35 g L^{-1} $FeCl_3$, 0.16 g L^{-1} $CuSO_4 \times H_2O$, 0.18 g L^{-1} $CoCl_2 \times 6$ H_2O, 0.01 g L^{-1} $NiCl_2 \times 6$ H_2O, 0.016 g L^{-1} $NaMoO_4 \times 2$ H_2O, 0.001 g L^{-1} $Na_2SeO_3 \times 5$ H_2O and was filter-sterilized with a 0.22 μm membrane filter (Carl Roth GmbH, Karlsruhe, Germany). The production media contained 5.0 g L^{-1} NH_4Cl, 14.6 g L^{-1} K_2HPO_4, 3.6 g L^{-1} $Na_2HPO_4 \times 2$ H_2O, 5.6 g L^{-1} Na_2SO_4, 0.54 g L^{-1} $MgCl_2 \times 6$ H_2O complexed with 1 g L^{-1} sodium citrate $\times 2$ H_2O, 0.26 g L^{-1} $CaCl_2 \times 2$ H_2O, 0.01 g L^{-1} thiamin and 5 g L^{-1} glucose. A total of 3 mL L^{-1} trace element solution and 100 mg L^{-1} ampicillin were added. The pH value was adjusted to 7.0 and was controlled during the cultivation using 4 M NaOH or 4 M H_3PO_4. To induce gene expression, 6 g L^{-1} of rhamnose was added to each bioreactor. Feed solution 1 contained 15 g L^{-1} $MgCl_2 \times 6$ H_2O and 7 g L^{-1} $CaCl_2 \times 2$ H_2O. Feed solution 2 contained 28.0 g L^{-1} NH_4Cl, 81.8 g L^{-1} K_2HPO_4, 10.1 g L^{-1} $Na_2HPO_4 \times 2$ H_2O, 15.8 g L^{-1} Na_2SO_4, 0.045 g L^{-1} thiamin and 150 g L^{-1} glucose; 30 mL L^{-1} of Trace element solution an 100 mg L^{-1} ampicillin were

TABLE 3 *Assessment of student learning comparing prelab (numbers in brackets) and postlab short written exams*

Question/task (category)	Outstanding	Reasonable	Needs improvement
A. Outline components and peripherals of a typical small-scale bioreactor system and place them in the right context.	Complete representation of all relevant components, functions assigned correctly 79 (5)%	Most components and functions assigned correctly 16 (11)%	Several vital components missing and mostly assigned the wrong function 5 (84)%
B. Explain offline and online analytics used in this experiment and outline how crucial fermentation parameters can be extracted from the online data.	Complete representation of applied online and offline analytics, principles of measurement explained and link to fermentation parameters (e.g. OTR) established; 32 (5)%	Mentioned most principles with clear description of methods, lacking description of link to fermentation parameters; 65 (19)%	Mentioned some analytics, inaccurate representation of methods, did not establish link to characteristic parameters; 4 (77)%
C. Explain the expression system and state what is required to produce recombinant protein with this system.	Complete representation of all relevant aspects (e.g. catabolic repression). Mentioned genetic setup and plasmid components; 14 (8)%	Most aspects explained, plasmid components explained to some extent; 83 (11)%	Description of fundamentals obscure, did not mention plasmid; 4 (82)%
D. Explain why different modes of operation or used for the experiment, and what purpose they serve.	Accurate division into batch and fed-batch mode, explanation why this is necessary, provided approximate reference values (e.g. growth rate, yields) for different stages; 44 (3)%	Accurate division into batch and fed-batch mode with basic explanation why this is necessary; 51 (9)%	Division into batch and fed-batch illustrated, explanation why this is necessary absent or incorrect; 5 (88)%

Presented data have been obtained from a total of 121 students from classes of 2013 (59 students) and 2014 (62 students). Sample questions for each category are available as Supporting Information S2. One representative question from each category assigned to learning objectives 3 to 6 (Table 1) is shown which has been rated according to the guidelines presented above.

added. The composition of the mineral salt medium was developed in-house, by referring to the average elemental composition of biomass. Detailed directions for the preparation of media and media components are provided as Supporting Information.

Bacterial Strain and Plasmid

Escherichia coli BW3110 [8] with plasmid pJoe4065.2 [9] was used for all cultivation experiments. The bacterial strain and plasmid was obtained by courtesy of Dr. Josef Altenbuchner (University of Stuttgart, Germany). The bacteria were stored routinely as glycerol stocks with 20% (*v/v*) glycerol at $-80°C$.

Cultivation in Shaking Flasks

All shake flasks were incubated in a shake incubator chamber (Multitron II, Infors AG, Bottmingen, Switzerland). For the first culture a total volume of 50 μL glycerol stock solution of the strain was used for the inoculation of 25 mL LB

in 100-mL shake flasks. This culture was incubated at $37°C$ and 120 rpm for 24 h. After 24 h a total volume of 1 mL was transferred to 500 mL shake flasks filled with 100 mL mineral salt media. The seed culture was incubated for 16 h, 120 rpm at $37°C$. For the inoculation of the bioreactor, a total of 25 mL of the seed culture was used to a final optical density ($\lambda = 600$ nm) of approximately 0.2.

Bioreactor System

The bioreactor system consisted of a 2.5 L glass vessel, with a maximum working volume of 1.5 L (Minifors, Infors AG, Bottmingen, Switzerland). Stirring was performed by a rushton-turbine, and gassing was controlled in the range of 0.1 to 4.0 Lpm. Temperature was maintained at $37°C$. All bioreactors were equipped with online exhaust gas analyzers BCP-O_2 and BCP-CO_2 (BlueSens gas sensor GmbH, Herten, Germany). Fermentation data were recorded by IRIS process control software (Infors AG, Bottmingen, Switzerland). For control of the fed-batch, external

peristaltic pumps equipped with pump heads (323Du and 314D, Watson-Marlow GmbH, Rommerskirchen, Germany) were used. Feeding was determined by recording the weight of the feed bottle, which was placed on a laboratory scale (model PCB 3500-2, Kern & Sohn GmbH, Balingen, Germany). Both the pump and the scale were connected to a personal computer running Microsoft Windows 7 via a RS232 serial connection. Control of the pump and feeding was performed in a numerical computing environment (MATLAB, The MathWorks, Natick, MA). The general setup of the fermentation system including bioreactor, exhaust gas analyzer, pumps and scale is shown in Fig. 2.

Calculations

Specific growth rates were calculated from sampling times and measured cell densities [Eq. (1)]. Out of practicality, the students determined the biomass concentration by measuring the optical density of the culture at $\lambda = 580$ nm, and a conversion factor for the estimation of cellular dry weight (CDW) from the optical density was provided by the supervisors (an OD_{580nm} of 1.0 usually corresponds to 0.33 g L^{-1} biomass concentration). After the fermentation, students calculated the correlation between optical density and cellular dry weight gravimetrically by drying a bacterial pellet from a defined amount of culture at 100°C overnight.

$$\mu = \frac{\ln\left(\frac{CDW(t_2)}{CDW(t_1)}\right)}{t_2 - t_1} \tag{1}$$

The substrate-to-biomass conversion yield ($Y_{X/S}$) was determined by calculation of cellular dry weight and substrate concentration at two given times [Eq. (2)], with V_{BR}: total volume of the culture [L] and c_S: concentration of glucose [g L^{-1}]. It should be noted that for the calculation of $Y_{X/S}$ in the fed-batch, the volume of feed added V_{Feed} [L] should be included for estimating the total volume in the bioreactor [Eq. (3)].

$$Y_{X/S} = \frac{CDW(t_2) - CDW(t_1)}{[c_S(t_1) - c_S(t_2)] \cdot V_{BR}} \tag{2}$$

$$Y_{X/S} = \frac{CDW(t_2) - CDW(t_1)}{c_S(Feed) \cdot [V_{Feed}(t_2) - V_{Feed}(t_1)]} \tag{3}$$

The oxygen transfer rate (OTR) is calculated from the gassing rate and the amount of oxygen as determined by exhaust gas analysis [Eq. (4)], with V_m: molar gas volume [mol L^{-1}], x_{O2}: mole fraction of oxygen in the exhaust gas and $\dot{V}$: gassing rate [L h^{-1}].

$$OTR = \frac{M_{O_2}}{V_{BR} \cdot V_m} \cdot [(x_{O_2,in} \cdot \dot{V}_{G,\ in}) - (x_{O_2,out} \cdot \dot{V}_{G,out})] \tag{4}$$

The calculated OTR is then used to determine the volumetric oxygen mass transfer coefficient (k_La), with c*: saturation concentration of oxygen in the media [mg L^{-1}] [Eq. (5)]. The saturation concentration was provided by the

instructor (7 mg L^{-1}), which can be approximated by referring to saturation concentrations of oxygen in a water-saline system at 37°C or measured by O_2-titration.

$$k_La = \frac{OTR_{max}}{c^* \cdot (1 - \frac{p_{O_2}}{100})} \tag{5}$$

The oxygen-to-biomass conversion yield is derived according to Eq. (6). This is performed by comparing biomass produced and oxygen transferred to the culture over a set amount of time.

$$Y_{X/O_2} = \frac{CDW(t_2) - CDW(t_1)}{(t_2 - t_1) \cdot OTR_{max} \cdot V_{BR}} \tag{6}$$

Student Instructions

Student instructions and guidelines, which contain detailed information on each individual operation, are supplied as Supporting Information (S3).

Faculty Instructions

Even though the general schedule of the course is predefined by the experimental design, several things may be adjusted as required. The following section is meant to give an overview on how the lab course was structured for the classes of 2012 to 2014. In addition, hints and tricks which can be helpful for the instructors are included in this section. Things that may require for a different schedule include a different state of previous knowledge of the audience, available equipment and personnel, the available timeframe or requirements for grading and examinations. Different possibilities for variations are addressed at a later point in this work. Specific details, e.g. the composition of the growth medium, are omitted in this section, and can be found in section "Materials and Methods." A general overview of the schedule, teaching concepts, and methods is illustrated in Fig. 1.

Personnel

The course is routinely performed with one instructor for each group of six students in the laboratory, and one teaching assistant for classroom tutoring. Both graduate students as well as undergraduate teaching assistants are supervising the course.

Preparation

Depending on the setup of the work stations, it may be favorable to reserve 1 to 2 weeks for preparation. It may be favorable to prepare the trace element solution and glucose solution in advance in a large batch for all students, since the concentrated glucose solution (50% w/v) required for the feed solution takes some time to dissolve properly. Depending on the number of students and stirrers available, this may consume time which can be used more productively otherwise. It may also prove beneficial to inoculate a backup culture in shaking flasks by the instructors, which can be used to replace a culture prepared by the students if required.

TABLE 4 *Assessment data obtained from in-lab interviews and evaluation of lab journals in terms of scientific writing capabilities (learning objectives 1 & 2, Table 1)*

Question/task	Outstanding	Reasonable	Needs improvement
Scientific analysis and documentation of data in the students individual lab journal.	Complete and correct analysis and interpretation of all fermentation data, advanced interpretation of characteristic parameters, outstanding scientific writing; 14%	Correct analysis and interpretation of most data, advanced calculations performed in part, acceptable scientific writing; 80%	Several calculations missing or incorrect, incomplete representation of data, no advanced calculations, scientific writing below average; 7%
Planning and execution of experimental procedures.	Well-organized planning and development of an experimental schedule, outstanding division of tasks and teamwork; 14%	Mostly well-organized planning, reasonable experimental schedule, workflow organized as a team; 78%	Planning and experimental schedule only in cooperation with supervisor, no teamwork evident; 9%

Presented data have been obtained from a total of 121 students from classes of 2013 (59 students) and 2014 (62 students). Students were rated according to the guidelines presented below. Details on the assessment are available as Supporting Information (S4).

Information in Advance

The students were informed 2 weeks before the lab course about what they would be required to prepare and bring. Safety instructions or introductory lectures if applicable may also be performed in advance to save time on first day in the lab.

Day 1. The first day of the lab course starts with a lecture on the basics of what the students will be required to know. Special attention here is given towards what equipment will be available for them and how each individual component works together. During the lecture, students are encouraged to actively participate while gathering relevant knowledge for the lab course, which is then collected on the board. This task is performed as a think-pair-share exercise, and usually results in a collection of details from different disciplines (e.g. requirements for growth, system for expression of recombinant protein, required equipment for cultivation, and measurements etc.). The first day mainly serves the purpose for the students to familiarize themselves with the equipment provided, and how it is used to perform the assignment. This includes the bioreactor with all peripheral devices like pumps, scales, pH and pO_2 sensors, stirrer type, and the exhaust gas analyzers. Each group of students is introduced to the bioreactor and how each individual part fulfills its function in a show-and-tell fashion by an instructor. Each group of students was then asked to perform the calibration of the pH-sensor, pO_2-sensor, and the peristaltic pumps. The students are encouraged to perform every step by themselves, thereby losing their inhibition towards the formerly mainly unknown equipment. In addition, the students start a blank fermentation without cells and medium to investigate how certain functions work, have an influence on other parameters and how they can be controlled (e.g. pH correction, effect of gassing, and stirring on dispersion of the air bubbles). Another vital part of this exercise is to learn how the process control software works as well as how data are collected, displayed and stored on the computer. The first day ends with the preparation of the first bacterial culture in shaking flasks, which can then be used on the next day to inoculate the seed culture for the bioreactor.

Day 2. The second day begins with a tutorial in which specific details on the experiment are discussed. This includes how medium, feed, acid and base are prepared, the setup of the bioreactor itself, and which steps are necessary prior to the sterilization of the bioreactor. For this task, the students are provided with a checklist, which summarizes important point that need to be followed (Supporting Information S3). Starting from this point, and from the knowledge gained on the previous day, the students are usually able to prepare the bioreactor for the experiments by themselves. Once the reactor is sterilized, everything is set-up in place and connected (e.g. acid/base). Special care is required for the supplementation of the culture medium with Mg/Ca solution. Instructors should make sure that it is added dropwise to the medium to avoid solubility issues that may distort measurement of optical density. Students are furthermore encouraged to familiarize themselves (e.g. by drawing) with the experimental scheme, which should summarize the processing of the samples and analytics. After this step is completed, the required tubes for the next day are labeled, and a sampling scheme is developed. The day in the lab ends with the inoculation of the seed culture, which is left to
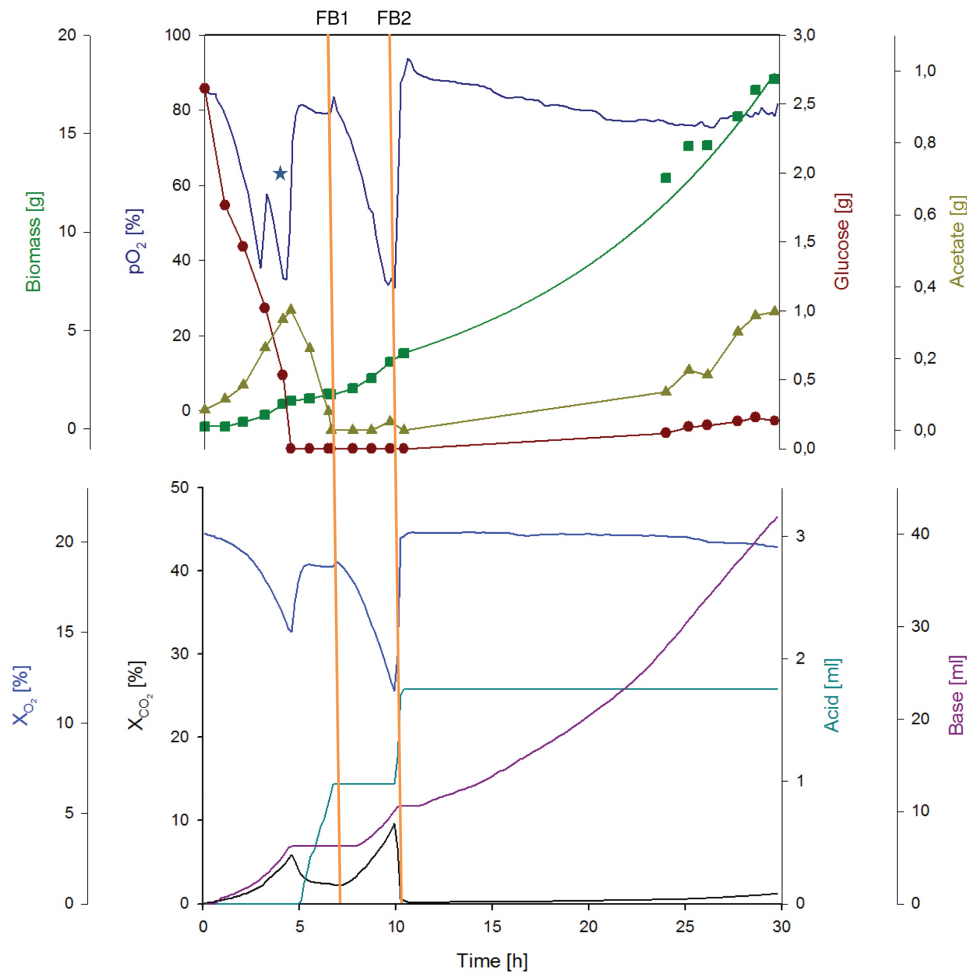
FIG 3 *Exemplary results from online and offline monitoring of the process. Online data are available for pO_2, x_{O2}, x_{CO2}, and the amount of acid and base added for adjustment of pH. Offline measurements as absolute values are provided for biomass, glucose, and acetate. Different modes of operation are indicated by orange vertical lines which indicate the transitions from batch phase (t = 0–6.5 h) to first fed-batch (t = 6.5–10.5 h) and second fed-batch (t = 10.5–30 h). Stirrer speed has been adjusted from 400 rpm to 500 rpm by process control software to enhance oxygen transfer to the culture broth at t = 3.5 h (indicated by a star), which results in a rise in pO_2 of no significance to the determination of substrate depletion. Biomass growth in the second fed-batch is approximated by assuming exponential growth with μ = 0.1 h^{-1} (dark green, solid line).*

growth overnight to be used as an inoculum for the bioreactor on the next day.

Day 3. The third day of the practical course is reserved for the actual experiment, the sampling procedures and the monitoring of the process. At this point, on the first 2 days of the course, everything should be set-up and prepared accordingly, so that the students can concentrate entirely on what happens in the experiment. Instructors should pay special attention to the inoculation, since it is best performed as fast as possible (within 5 min after removing the culture from the incubator), to avoid longer lag phases. The students are furthermore interviewed in front of the bioreactor, to assess how well they were able to absorb the

theoretical knowledge from the lecture, and how well they can connect it to real-life biotechnology (Fig. 1, Table 1). The experiment is left to run overnight, with automatic feeding protocols put in place, to continue sampling on the next day. Feeding is performed with MATLAB, and the program code is available as Supporting Information.

Day 4. Once the last samples from the bioreactor are processed, the experiment is finalized by switching off the reactor and the collection of data. The bioreactor is then prepared for sterilization once more, according to the checklist that has been handed to the students on day 2. During the time required for sterilization, a tutorial on interpretation and analysis of the collected data is provided

for the students. After that once the reactor has cooled down, it can be disassembled and cleaned. After reassembly of the bioreactor, the rest of the day is reserved for calculations and analysis.

Day 5. The last day of the lab course starts with a short written exam, which is meant to assess the students progress in the lab course. If required, this day may also serve as a buffer, in case some analytics could not yet be performed, or if the students did not finish cleaning and reassembling the bioreactor yet. At the end of the day, the students are asked to hand in their lab reports.

Safety Issues

The entire practical work is performed in a biological laboratory, which requires for appropriate safety measures. The strain used belongs to biosafety group 1, and can therefore be regarded as generally non-pathogenic for humans. All chemicals used are non-toxic. A constant titration of the culture media with acid and base induces a need to wear safety glasses and lab coats at all time. In addition, during control of a potential leakage of the bioreactor, the system is pressurized. The exhaust air filter may, in rare cases, become clogged due to foaming of the culture, which results in increased pressure in the bioreactor vessel, therefore reinforcing the need to wear safety glasses. Safety gloves are mandatory if acid and base are handled, otherwise they can be used as required. Students are made aware of these facts in advance in a prelab safety briefing. In general, understanding and following safety regulation in a biological laboratory can be regarded as an additional important learning objective.

Discussion

Field Testing

The practical course is performed once a year, generally in three 1-week session with 18 to 24 students each week. Students are teamed up with a partner randomly, to promote the formation of interdisciplinary teams. Each group of two students is assigned to a workstation with a bioreactor. In this section, data from classes of 2012 to 2014 are presented, with a total number of 178 students: 57 (2012), 59 (2013), and 62 (2014), respectively. Students' subjective opinion and perception of the course was evaluated. This has been performed within the frame of an official anonymous routine evaluation of university teaching of the German state of Baden-Wuerttemberg. The results are presented in Fig. 5 and includes both students own assessment of learning outcomes as well as level of the course and teaching quality.

Students were asked to answer each question on a scale from 1 to 5 (e.g. from 1—"very clear" to 5—"not at all"), as described in Fig. 5. Students reported that the course was meaningful to them and their studies (questions 1 and 3) and that both preliminary knowledge required as well as the level of the course were slightly above average

(questions 4 and 6). In addition, more than 90% of students from all classes reported a gain in knowledge, choosing either "1—completely true" or "2—mostly true" (question 5). Comparing the results from the evaluation, student's overall perception and acceptance of the lab course is positive throughout the classes of 2012-2014.

Evidence of Student Learning

Students from classes of 2013 and 2014 participated in short written prelab and postlab exams. In addition, every participant was required to submit a lab journal upon completion of the course, and students' practical skills were monitored and assessed during their time in the lab.

Comparing the data obtained by written exams, the learning outcome of the course could be assessed (Table 3). Prelab written exams were performed anonymously by randomly generated tests containing at least two questions from each category according to Table 1. Postlab written exams were structured similarly and were performed on the last day of the course. A summary of potential questions, which may be used for this purpose, are provided as Supporting Information (S2). Students' answers were then rated for each category, and classified as either "outstanding," "reasonable," or "needs improvement." Data from Table 3 show that in all four categories (corresponding to four learning objectives, Table 1), at least 95% of the participants showed at least satisfactory learning outcome (ranked as either "outstanding" or "reasonable") in postlab exams. In comparison, this was only achieved by less than one fourth of the students in all categories of prelab exams.

Additionally, criteria have been developed to classify students' behavior during the practical part of the experiment. These criteria, besides general organization as mentioned above, include students' ability to engage in teamwork, as well as cleanliness of the workstation, abilities which are otherwise very hard to assess during written exams. The main criteria for classification are provided in Table 4, and a detailed list can be found in Supporting Information S4. The assessment was performed by regular informal in-lab interviews with associated tentative questioning by the supervisors. Using this method, instructors could accompany the experiment without conveying too much pressure on the students. Therefore, difficulties in understanding of individual participants could be identified early and addressed properly, which was found to be suitable tool to account for a potential variable background of the students. According to this procedure, students learning outcome in the practical part was classified as either "outstanding," "reasonable," or "needs improvement." The results of this classification are shown in Table 4. Approximately 92% of students displayed at least satisfactory behavior and skills.

Student's lab journals, which contained data and calculations as well as experimental descriptions, were evaluated to assess students understanding of the complex background and scientific writing skills. The assessment
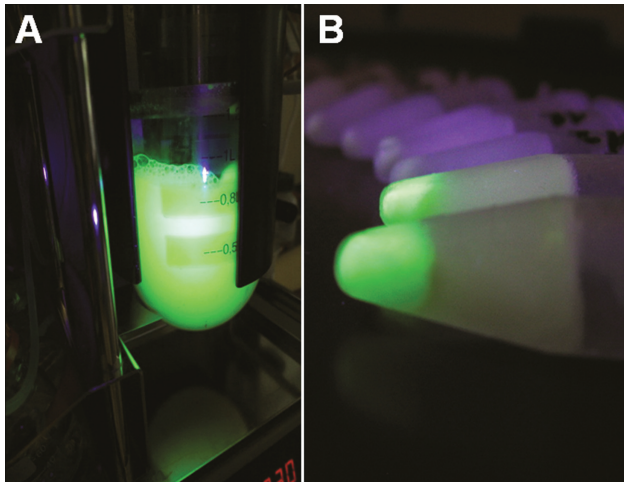
FIG 4 eGFP-fluorescence at the end of the fermentation. (a) eGFP-producing bacteria can easily be visualized in the glass vessel of the bioreactor by using UV light. (b) The process of eGFP-production during the fermentation becomes apparent by comparing frozen cell-pellets under UV light.

focused on correctness and understanding of necessary calculations and interpretation of common characteristic parameters as well as the ability to put the content of the course in a correct context. The comparison of these results to values common to other bioreactor systems or bioprocesses is meant to enhance students' critical-thinking skills. The classification was performed according to criteria in Table 4, and ranked as explained above. Approximately 94% of students displayed at least satisfactory results.

Sample Data

During the experiment, several online and offline parameters had to be monitored by the students. These include time course measurements of biomass (c_X), online monitoring of dissolved oxygen (pO_2), glucose (c_{glc}), and acetate (c_{ac}) concentration, exhaust gas analysis (x_{O2}, x_{CO2}) as well as acid and base volumes added to the bioreactor for pH control. A typical set of results is depicted in Fig. 3. In batch mode, the biomass increases exponentially from the initial value at the point of inoculation of approximately 0.1 g to a total of approximately 1.5 g once the initial amount of 2.5 g glucose is depleted. Due to overflow metabolism, acetate is produced and accumulates, respectively. After the end of the batch process on glucose, the consumption of acetate accounts for a slower growth rate, represented by a smaller gain in biomass between $t = 5$ h and $t = 6.5$ h. The growth profile in batch mode is furthermore shown by the online parameters pO_2, x_{O2}, and x_{CO2}. During growth on glucose ($t = 0$–5 h), pO_2 as well as x_{O2} decline while x_{CO2} increases, as biomass is produced. The increase in pO_2 at $t = 4$ h (Fig. 3, indicated by a star) corresponds to an increase in stirrer speed, which has been automatically adjusted by the process control software to prevent oxygen limitation. Upon adjustment of the metabolism from glucose to acetate at $t = 5$ h, pO_2 as well as x_{O2} increase, due to the slower metabolic rate. The time course of x_{CO2} follows x_{O2} in an opposite way, since less O_2 consumed causes less CO_2 to be produced. The third increase in pO_2 at $t = 6.5$ h indicated the depletion of acetate, and therefore the starting point for the first fed-batch. During the exponential growth phase, ammonia is consumed as the only available source of nitrogen, which results in a need for pH adjustment. Base is added to maintain a constant pH, which stops once glucose is depleted. The consumption of acetate leads to the addition of acid ($t = 5$–6.5 h), respectively.

During exponential feeding in the first fed-batch (FB1, $t = 6.5$–10.5 h) pO_2, x_{O2}, x_{CO2} and base levels behave as described for the batch process during glucose consumption. To maintain a constant growth rate of $\mu = 0.5$ h^{-1}, limiting amounts of glucose are fed resulting in limiting concentrations of glucose in the fermentation broth. The addition of acid at the end of the first fed-batch process may be attributed to the degradation of small levels of acetate which formed during feeding.

The second fed-batch begins at $t = 10.5$ h, and the growth rate is set to $\mu = 0.1$ h^{-1} by adjusting the feeding rate. This decrease in growth rate is represented by a slower formation of biomass and lower amount of glucose fed to promote cAMP formation. This, besides the induction with rhamnose, which is added at the beginning of the second fed-batch, is a prerequisite for the production of recombinant eGFP (refer to section "Scientific background"). Additionally, at that point, the gassing rate is increased to eliminate the risk of oxygen limitation at higher cell densities, since this step is usually performed overnight. The increase in pO_2 at the beginning of the second fed-batch (FB2, $t = 10.5$–30 h) can be attributed to the increase of gassing rate, the decreased temperature as well as the lower growth rate. No distinct changes in x_{O2} and x_{CO2} can be observed during the second fed-batch due to the higher gas flow rate.

Regarding the product yield, it is especially important to control the addition of feed solution during the early production phase (fed-batch 2), where incorrect dosage of feed solution will cause a buildup of both glucose and acetate, thereby dramatically lowering final eGFP yield. The adjustment of temperature from 37°C to 30°C, which is typically performed in experiments with inducible protein expression to enhance the amount of correctly folded, active protein, only had a small effect on final levels of active eGFP. However, due to didactic reasons, this temperature shift was not removed from the procedure.

The visual result of the experiment, the fluorescence of eGFP is visualized under UV both in the bioreactor as well as the biomass pellets (Fig. 4). At the end of the second fed-batch, at $t = 25$–30 h, an accumulation of glucose and acetate can be observed. The increase in glucose and
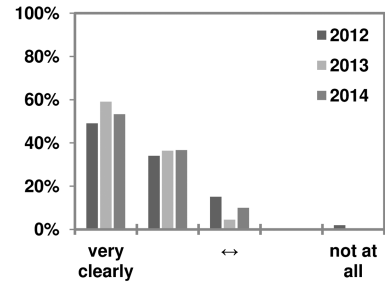
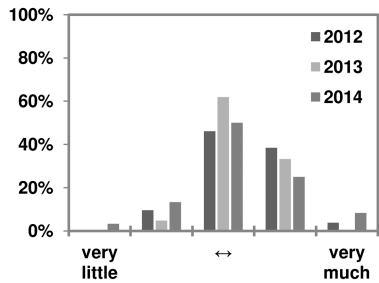**Did you gain insights relevant to your desired profession?**
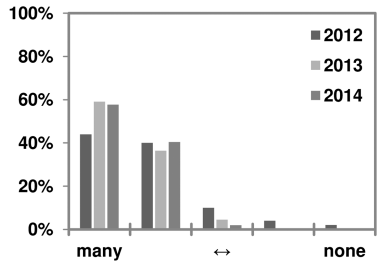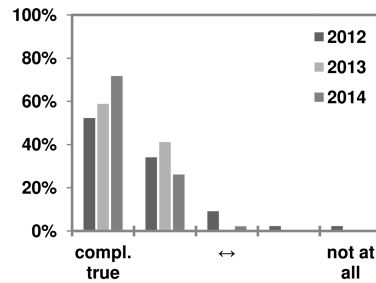
Experimental descriptions were …
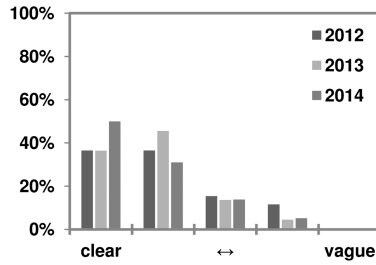
Do you understand the importance of the course to your further studies?

Preliminary knowledge required was …

I learned a lot during this course

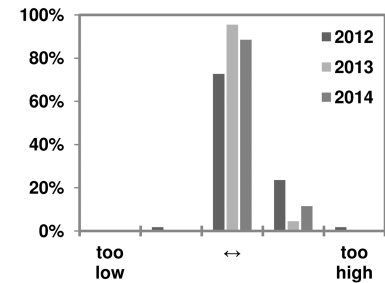**The level of the course was …**

FIG 5 Student perception on their learning experience during the lab course. Presented data have been collected within the frame of the official evaluation of higher education at the Karlsruhe Institute of Technology (KIT), in a program established at all universities of the German state of Baden-Wuerttemberg. Evaluation data were collected anonymously in written form on the last day of the lab-course. The amount of students participating in the study were 57 (2012), 59 (2013), and 62 (2014), respectively.

acetate levels may be attributed to transport and mixing issues occurring at high cell densities. An inhomogeneous culture may lead to high local concentrations of glucose which triggers the formation of acetate. Additionally, local gradients may occur due to drop-wise feeding of glucose. Furthermore, sampling reduces the total volume of the culture, which is not covered by feeding control. Over time, this may result in higher amounts of glucose being added to the reactor, leading to a potential accumulation of acetate.

Based on online and offline measurements, key process parameters are calculated and interpreted by the students. These parameters include the total biomass, maximum growth rate (μ_{max}), biomass yields for glucose and oxygen ($Y_{X/glc}$, $Y_{X/O2}$), oxygen transfer rate (OTR), and the volumetric oxygen mass transfer coefficient (k_La). Sample results and typical ranges are summarized in Table 2.

Possible Modifications

The lab course, as described within this work, requires for a specific setup of equipment and peripherals besides the bioreactor. However, there is room for significant variations, both in terms of timeframe and scope as well as with respect to the available equipment. For example, if no exhaust gas analyzer is available, a dynamic method can

be employed for the calculation of OTR, which does not require for online exhaust gas monitoring. The course can easily be extended to a 2 or 3-week session, which may be achieved by broadening the scope. Thereby, other disciplines like genetics and molecular biology gain a stronger representation in the lab course. One way could be the integration of genetic engineering in week 1, where the students are preparing the bacterial strain for use in the experiment on the next week. This could include the amplification of the target gene by PCR, the restriction digest of the appropriate plasmid and gene, as well as ligation and transformation of the construct. The week after the production of recombinant protein in the bioreactor could be used for downstream processing. This may be performed using purification by affinity chromatography with tagged proteins. The construct used in this lab course leads to expression of 6xHis-tagged eGFP on the C- and N-terminus, which can be purified by immobilized metal affinity chromatography (IMAC). The purity of the obtained fractions during purification may then be determined by SDS-PAGE with subsequent protein staining, or by quantification using fluorescence readings combined with determination of total protein content. Another potential modification of the lab course would be an alteration of the tasks towards an engineering perspective. The setup of the experiment is suitable

for an exercise in the field of control engineering, e.g. the development and programming of appropriate controllers or software, e.g. for the feeding process.

Conclusion

In this work, the theory, application and assessment of a lab course in bioprocess engineering for undergraduate students has been presented and discussed. It is demonstrated that the course is very flexible, robust and easy to reproduce, and can be held even if only basic equipment and bioreactors are available. Therefore, this study can be beneficial for instructors in biotechnology or related disciplines and provides a basis for implementing or reworking a lab course in biotechnology. Additionally, several variations were presented to tailor the course to fit in a broader curriculum. The course is aimed at teaching bioprocess engineering in a motivating and active multidisciplinary environment, which benefits students teamwork and communication skills. Solving real-life problems and questions benefits students ability to critically evaluate the experimental design and outcome. Along with the framework of the course, learning objectives have been developed to cover all relevant aspects of the subject matter, and the results from assessment of student learning demonstrate that the desired learning outcome could be reached.

Acknowledgments

References

[1] Biggs, J. B. (2003) Teaching for Quality Learning at University, 2nd ed., Society for Research into Higher Education and Open University Press, Buckingham.

[2] Bloom, B. S. (1956) Taxonomy of Educational Objectives; The Classification of Educational Goals, Longman Group, New York.

[3] Duch, B. J., Groh, S. E., and Allen, D. E. (2001) The Power of Problem-Based Learning: A Practical "How to" for Teaching Undergraduate Courses in any Discipline, Stylus Publishing, Sterling, VA.

[4] Handelsman, J., Miller, S., and Pfund, C. (2006) Scientific Teaching, 1st ed., W.H. Freeman, New York.

[5] Shimomura, O., Johnson, F. H., and Saiga, Y. (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea, J. Cell. Comp. Physiol. 59, 223-239.

[6] Matthews, J. C., Hori, K., and Cormier, M. J. (1977) Purification and properties of *Renilla reniformis* luciferase, Biochemistry 16, 85-91.

[7] Patterson, G., Day, R. N., and Piston, D. (2001) Fluorescent protein spectra, J. Cell. Sci. 114, 837-838.

[8] Hill, C. W. and Harnish, B. W. (1981) Inversions between ribosomal RNA genes of *Escherichia coli*, Proc. Natl. Acad. Sci. USA 78, 7069-7072.

[9] Wegerer, A., Sun, T., and Altenbuchner, J. (2008) Optimization of an *E. coli* L-rhamnose-inducible expression vector: test of various genetic module combinations, BMC Biotechnol. 8.

[10] Wilms, B., Hauck, A., Reuss, M., Syldatk, C., Mattes, R., Siemann, M., and Altenbuchner, J. (2001) High-cell-density fermentation for production of L-N-carbamoylase using an expression system based on the *Escherichia coli* rhaBAD promoter, Biotechnol. Bioeng. 73, 95-103.

[11] Wayne, P. K. and Rosen, O. M. (1974) Cyclic 3':5'-adenosine monophosphate in *Escherichia coli* during transient and catabolite repression, Proc. Natl. Acad. Sci. USA 71, 1436-1440.

[12] Lemuth, K., Hardiman, T., Winter, S., Pfeiffer, D., Keller, M. A., Lange, S., Reuss, M., Schmid, R. D., and Siemann-Herzberg, M. (2008) Global transcription and metabolic flux analysis of *Escherichia coli* in glucose-limited fed-batch cultivations, Appl. Environ. Microbiol. 74, 7002-7015.

[13] Hardiman, T., Lemuth, K., Keller, M. A., Reuss, M., and Siemann-Herzberg, M. (2007) Topology of the global regulatory network of carbon limitation in *Escherichia coli*, J. Biotechnol. 132, 359-374.

[14] Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*, J. Bacteriol. 62, 293-300.