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Simple defined autoinduction medium for high level recombinant protein production using T7-based *E. coli* expression systems

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Abstract

Protein production under the control of *lac* operon regulatory elements using autoinduction is based on diauxic growth of *E. coli* on lactose after consumption of more preferred carbon substrates. A novel simple and cost-effective defined autoinduction medium using a mixture of glucose, glycerol, and lactose as carbon substrate and NH_4^+ as sole nitrogen source without any supplementation of amino acids and vitamins was developed for T7-based *E. coli* expression systems. This medium was successfully employed in 96-well microtiter plates, test tubes, shake flasks and 15 liter bioreactor cultivations for production of different types of proteins achieving an average yield of 500 mg L^{-1} product. Cell specific protein concentrations and solubility were similar as during conventional IPTG induction using Luria Bertani broth. However, the final yield of target proteins was about 4 times higher as a higher final biomass was achieved using this novel defined autoinduction broth.

Key words: *Escherichia coli*, recombinant protein production, autoinduction; defined medium.

Introduction

The gram-negative bacterium *Escherichia coli* is the most popular organism for recombinant protein production because of its well-characterized genetic background, its ability to grow to high cell densities, and the large number of available cloning vectors and optimized host strains. Among numerous *E. coli* based expression systems, the bacteriophage T7 RNA polymerase controlled system is currently the most popular protein production system (Studier and Moffatt 1986). The T7 RNA polymerase is usually under control of the *lac* operon, thus protein production initiated by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG).

The idea of autoinduction was introduced as a convenient way to perform recombinant protein production without inducer addition in small laboratory scale for *lac* operon controlled expression systems (Studier 2005). Carbon substrate mixtures of glucose, glycerol and lactose are used, where glucose as the preferred carbon substrate is utilized first, followed by glycerol and lactose which also acts as the inducer of *lac* operon controlled protein production (Blommel et al. 2007).

Autoinduction omits biomass monitoring for the correct timing of inducer addition and places the shift from growth to recombinant protein production under metabolic control of the expression host (Blommel et al. 2007).

There are several complex autoinduction media currently available: the original medium described by Studier (Studier 2005) and others from commercial sources (Overnight Express™ Autoinduction System from Novagen and MagicMedia™ from Invitrogen with unknown ingredients). The presence of complex medium compounds such as tryptone, yeast extract and others render these media expensive and also not compatible with protein labeling (selenomethionine or heavy isotope labeling). Thus, several defined autoinduction media have been described later but all contain various amino acid and vitamin supplements (Blommel et al. 2007; Fox and Blommel 2009; Sreenath et al. 2005; Sivashanmugam et al. 2009; Tyler et al. 2005). For example, a defined autoinduction medium using an enhanced GFP as a reporter protein was announced which contains 18 different amino acids, 8 different vitamins, and 10 different trace elements; altogether 46

different compounds (Blommel et al. 2007; Fox and Blommel 2009). This medium was successfully employed for screening studies in 96-well microtiter plates (Frederick et al. 2007), but lower yields were achieved in larger-scale culture vessels (Blommel et al. 2007). Also, this medium has only been tested for a few proteins (Bitto et al. 2008; Bitto et al. 2009; Oppenheimer et al. 2010; Sobrado et al. 2008) and, thus, has not been proven for universal applicability.

Here, we present a novel, simple-to-prepare defined autoinduction medium consisting of glucose, glycerol and lactose as carbon substrate and NH_4^+ as sole nitrogen source. Supplementation of this medium with amino acids and vitamins is not required. This medium was developed using three target proteins with different properties [Green fluorescent protein (GFP), glutathion-S-transferase-tagged GFP, and human basic fibroblast growth factor] and optimized in test tube and shake flask cultures. Finally, it was successfully tested for production of various proteins in the molecular mass range of 18 to 90 kDa. Moreover, the optimized autoinduction medium revealed the same efficacy for protein production in 96-well microtiter plates as well as in 15 liter bioreactor cultivations leading to high final biomass concentrations ($\text{OD}_{600} > 20$) and average yields of 500 mg L^{-1} target protein.

Materials and methods

Strains and plasmids

Strains and plasmids are described in Table 1. The construction of the plasmids pET-28c-His6-GFP and pETM30-His6-GST-GFP for production of different green fluorescent proteins, His6-GFP (abbreviation: GFP) and His6-GST-GFP (abbreviation: GST-GFP), under the control of the T7 promoter was as follows. The plasmid pBAD-GFPuv (Clontech, Heidelberg, Germany) was used to generate pET-28c and pETM30 vector combinations for the C-terminal fusion of GFP to proteins of interest. Site-directed mutagenesis was used to remove the internal *Nco*I, *Nde*I and *Bam*HI sites from the coding sequence of GFPuv. The reaction was done using the Quickchange Kit of

Stratagene as recommended by the supplier. In subsequent rounds of mutagenesis using the primer pairs GFPmut1 and mut2 (GFPmut1: 5'-GAAAACTACCTGTTCCCTGGCCAACACTTGTCA-3', GFPmut2: 5'-TGACAAGTGTTGGCCAGGGAACAGGTAGTTTTC-3'); GFPmut3 and mut4 (GFPmut3: 5'-CCGTTATCCGGATCACATGAAACGGCATGAC-3', GFPmut4: 5'-GTCATGCCGTTTCATGTGATCCGGATAACGG-3'); GFPmut5 and mut6 (GFPmut5: 5'-CACAACATTGAAGATGGAAGCGTTCAACTAGCAGA-3', GFPmut6: 5'-TCTGCTAGTTGAACGCTTCCATCTTCAATGTTGTG-3'), respectively, the restriction sites were removed. An N-terminal adapter sequence was introduced to be able to generate in frame fusions to genes of interest. The primer pair GFPfus1 and GFPfus2 (GFPfus1: 5'-CATGTGCCATGGGTCATATGTGTAACTGAGTAGGATCCGCTAGCGCTGGCTCCGCTGCTGGTTCTGGTA-3', GFPfus2: 5'-CTAGTACCAGAACCAGCAGCGGAGCCAGCGCTAGCGGATCCTACTCAGTTAACACATATGACCCATGGCA-3') was annealed and ligated in pBADGFPmut digested with *NheI*. Correct linker integration concomitant to removal of the downstream *NheI* site was verified by sequencing. After selection of the correct clone the vector was named pBADGFPFUS. The GFPFUS sequence was then cloned into pETM30 vector (EMBL, Heidelberg, Germany) using the *NcoI* site from the new linker and the downstream *EcoR1* site. The GFPFUS fragment was cloned into linearized pETM30 digested with *NcoI* and *EcoR1*. The positive clone was named pETM30GFPFUS. A similar clone pET-28c was generated by using the *NdeI*-*EcoR1* fragment of pBADGFPFUS cloned into the *NdeI* and *EcoR1* sites of pET-28c. This vector was named pET-28cGFPFUS. To create an in frame fusion of GFP to His6-GST, the vector pETM30GFPFUS was digested with *NdeI* and *BamH1*. The ends were filled using Klenow polymerase and dNTPs and thereafter the vector was religated. The resulting vector was called pETM30-His6-GST-GFP. The pET-28cGFPFUS vector was used to create a His-tagged GFP fusion by digestion with *NheI* and religation. This vector was called pET-28c-His6-GFP. The His6-GST-GFP (abbreviation: GST-GFP) and His6-GFP (abbreviation: GFP) expression vectors were used as model systems to analyze the efficiency of

recombinant gene expression.

Medium preparation

The composition of Luria Bertani (LB) broth (low salts) was as follows: 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, and 5 g L⁻¹ NaCl. The pH was adjusted to pH 7 using 5 mol L⁻¹ NaOH before autoclaving. For solidification 15 g L⁻¹ agar was added. The defined medium is based on methods described by Korz et al (1995), details in composition are given in Table 2. Special instructions for medium preparation are given in the Supplementary Tables 1 and 2. Briefly, trace elements (CoCl₂·6H₂O, MnCl₂·4H₂O, CuCl₂·2H₂O, H₃BO₃, Zn(CH₃COOH)₂·2H₂O and Titriplex III) were prepared as 2,000 times concentrated stock solution mixture, sterilized by filtration (0.2 µm) and stored at 4°C. Na₂MoO₄·2H₂O was prepared as 5,000 times concentrated stock solution, sterilized by filtration (0.2 µm) and stored separately at 4°C to prevent coprecipitation with the other trace elements during long term storage. Carbon sources and MgSO₄ (Glucose, glycerol, lactose and MgSO₄) were prepared together as a combined stock solution and sterilized by filtration (0.2 µm). For larger amounts, the combined glucose, glycerol, lactose and MgSO₄ stock solution was heat-sterilized (121°C, 20 min). After sterilization of the combined glucose, glycerol, lactose and MgSO₄ stock solution, the 5,000 times concentrated Na₂MoO₄·2H₂O stock solution can be added and the mixture can be kept at room temperature for long term storage (carbon source stock). Ammonium and phosphate salts additionally including citric acid and ferric citrate [(NH₄)₂HPO₄, KH₂PO₄, Citric acid and Fe(III)citrate] were prepared as combined stock solution. After adjusting the pH value to pH 6.8, the ammonium and phosphate salt stock solution can be heat-sterilized (121°C, 20 min) or sterilized by filtration (0.2 µm). When the pH is adjusted by ammonium hydroxide (30%), filter sterilization is preferable. After sterilization, the ammonium and phosphate salts stock solution can be mixed with the 2,000 times concentrated trace elements stock solution for long term storage at room temperature (salt-trace stock). Directly before inoculation the two major stock solutions (carbon source stock and salt-trace stock) were combined and supplemented with the appropriate

antibiotics. Antibiotics working and stock solutions were prepared according to “Molecular Cloning: A Laboratory Manual (Third Edition)” (Sambrook and Russell 2001). For bioreactor cultures, the pH was adjusted to pH 6.8 with ammonium hydroxide after heat-sterilization.

Analysis of cell growth

Cell growth was monitored by measurement of the absorbance at 600 nm (OD₆₀₀) and cell dry mass (DCM) as described (Korz et al. 1995). One unit of OD₆₀₀ corresponds to 0.37 gram dry cell mass per liter for *E. coli* BL21(DE3) [determined during growth in Defined Non-inducing Broth (DNB) at 30°C].

Analysis of protein concentration

Culture samples were centrifuged at 17,000 g and 4°C for 3 min. After removal of the supernatant, cell pellets were stored at -80°C until further analysis. For preparation of cell extracts and determination of soluble and insoluble product fractions, cell pellets were disrupted by BugBuster™ Protein Extraction Reagent (Novagen, USA) with rLysozyme and Benzonase according to manufacturer's instructions. Soluble and insoluble cell fractions were separated by centrifugation at 17,000 g and 4°C for 25 min. SDS-PAGE analysis of total cell protein, and soluble and insoluble protein fractions was performed using Criterion™ Tris-HCl Gel (8–16% polyacrylamide gel, 26-well) in Criterion™ Cell (Bio-Rad, USA) according to standard procedures (Bollag et al. 1996) and manufacturer's instructions. Electrophoresis was carried out at constant voltage (160 V). After electrophoresis, SDS-PAGE gels were stained using colloidal Coomassie G-250 (Candiano et al. 2004). Destained wet gels were scanned using ScanMaker 9800XL (Microtek, Taiwan). Expression levels of target protein (in percentage of total cell protein) were determined by AIDA Image Analyzer (Raytest, Germany) from SDS-PAGE. Specific and volumetric product concentrations were calculated assuming a constant cell protein content of 550 mg protein per gram cell dry mass (Ingraham et al. 1983). Fluorescence measurements were conducted in triplicate using

InfiniteTM M200 (Tecan, Switzerland) 96 well plate reader with 395 nm excitation and 510 nm emission filters as described (Albano et al. 1996) and according to equipment manufacturer's instructions.

Cultivation conditions

Test tube and shake flask cultivations. Test tubes with diameter of 10 mm, 100 ml or 250 ml Erlenmeyer flasks with three baffles and 1.8 L Fernbach flask with three baffles were used with working volumes of 2 ml, 10 or 25 ml, and 200 ml, respectively. Glycerol stocks of recombinant *E. coli* strain were streaked on LB agar plates and incubated overnight at 37°C. After overnight incubation, single colonies were picked and transferred to LB medium (2 ml in test tube or 10 ml in 100 ml shake flask). The cultures were shaken at 37°C for 4~6 h until the OD600 reached about 1.0. Following, DNB medium in shake flasks were inoculated with LB medium pre-cultures to give a starting OD600 of 0.02. Cultures were shaken at 37°C for 6~7 h until the OD600 reached 1.5~2.0. DNB medium pre-cultures were used to inoculate the main cultures (DNB or S-DAB) giving a starting OD600 of 0.02. The shaking speed was 180 rpm and 140 rpm, respectively, for test tubes and shake flasks using a shaker with an amplitude of 5 cm. The cultivation time using autoinduction medium was as follows: 18 h at 37°C, 26 h at 30°C and 48 h at 23°C. For IPTG induced cultivation (in LB or DNB medium), the inoculated cultures were shaken at 37°C until the OD600 reached 1.0 (LB medium) or 1.5 (DNB medium). Then 0.25 mmol L⁻¹ IPTG was added to the culture to start protein production and the temperature was reduced to 30°C or 23°C depending on the required induction temperature. The time of induction at 37°C, 30°C and 23°C was 4 h, 6 h, and overnight (about 16 h), respectively. Special attention should be paid to *E. coli* Rosetta 2 (DE3) which grows slower than *E. coli* BL21 (DE3). In this case, the starting OD600 value should be raised to 0.04 or the cultivation time prolonged. Test tube and shake flask cultivations were carried out in triplicate and duplicate, respectively. Mean values are given.

Bioreactor cultivations. For a 10 L autoinduction cultivation carried out in a 15 L bioreactor (Fermenter B10, Fa. Biologische Verfahrenstechnik, Switzerland) the following settings were chosen: starting OD600 of 0.02, temperature of 23°C, starting pH 6.8 (controlled at pH 6.8 by addition of ammonium hydroxide or non-controlled), air flow rate of 1 vvm, stirrer speed of 450 rpm. The conditions were kept constant during the entire cultivation. Off-gas analysis was performed using a BCproFerm system (BlueSens, Germany). The end of the cultivation was indicated by a sudden rise in the dissolved oxygen concentration indicating depletion of all carbon compounds.

Microtiter plate cultivations. For autoinduction cultivation carried out in 96 deep well plates (2 ml total volume per well), single colonies were transferred from LB agar plates to 0.5 ml LB broth (per well) and shaken at 1,000 rpm with an orbital amplitude of 2 mm at 37°C for 7 h to reach stationary phase. 5 µl of these LB pre-cultures were inoculated to 0.5 ml DNB (per well) and cultivation carried out overnight (16 h) at 25°C. After overnight cultivation, OD600 values of DNB pre-cultures were analyzed. Following, 0.5 ml of S-DAB (HNC) (per well) were inoculated with DNB grown pre-cultures to give a starting OD600 of 0.02. The cultures in 96 deep well plates were shaken at 25°C for 40 h. All cultivations were carried out in triplicate. Mean values are given.

Results

Choice of reporter proteins

For the development of a universal production medium with a broad application range three reporter proteins were chosen for medium optimization covering different protein properties, e.g. propensity to form inclusion bodies, size, biological activity, post-translational oxidative fluorophore formation.

Two fluorescent proteins were employed. The amount of properly folded green fluorescent protein

(GFP) can be easily accessed by fluorescence measurements rendering GFPs as the most popular reporter proteins (Chalfie et al. 1994; Tsien 1998). In addition to conventional GFP carrying an N-terminal His6 extension, a GFP fusion protein was chosen which additionally included the sequence of glutathion-S-transferase (GST) between the N-terminal His6 tag and GFP. The additional presence of the GST tag increases the size of the protein from 27 to 54 kDa and also strongly increases the propensity to form inclusion bodies. The GST-GFP fusion protein only emits fluorescence when the entire fusion protein is correctly folded. As third reporter protein human basic fibroblast growth factor (hFGF-2, 18 kDa) was employed. hFGF-2 can be produced as soluble active protein but also aggregates into non-active inclusion bodies. The expression level and the fraction of soluble active protein is strongly influenced by the culture conditions (Hoffmann et al. 2004; Hoffmann and Rinas 2000; Rinas et al. 2007).

The influence of temperature and carbon substrate composition on protein production using autoinduction

Screenings were carried out in test tube and shake flask cultivations and not in microtiter plates to prevent potential inaccuracies during autoinduction medium development resulting from evaporation (Blommel et al. 2007; Faessel et al. 1999).

The initial screening for temperature effects was carried out in test tube cultures using GFP and GST-GFP as reporter proteins. Both proteins were produced in test tube cultivations at 37°C, 30°C and 23°C employing a carbon source matrix with 9 independent combinations of glucose, glycerol and lactose (indicated as T1 - T9 in Table 2) and using IPTG induction in DNB as control.

A representative result showing the temperature effect on protein production using Defined Autoinduction Broth (DAB) is shown in Fig. 1. The volumetric product concentrations were not significantly affected by the temperature, however the amount of correctly folded protein (indicated as fluorescence per nM of GFP or GST-GFP) increased dramatically for both proteins with decreasing temperature. Thus, further experiments for optimizing the medium composition were

carried out at 23°C.

To obtain a general optimized autoinduction medium the carbon source matrix from test tube cultivations was complemented by a matrix including 12 different carbon source combinations employed in shake flask cultivations (S1 - S12) resulting in 18 different combinations of glucose, glycerol and lactose and containing 3 combinations which were tested both in test tube and shake flask cultures (Table 2). The carbon source combination screening was performed with the two fluorescent proteins and hFGF-2.

The results revealed that each protein has its unique optimal carbon source combination (Fig. 2). For example, the highest volumetric fluorescence of GFP was obtained in autoinduction medium without glucose. Increasing glucose concentrations were accompanied with decreasing volumetric fluorescence of GFP in agreement with previously reported results (Blommel et al. 2007). However, the best production of the GST-GFP fusion protein was achieved in the presence of $\sim 3 \text{ g L}^{-1}$ glucose and the best hFGF-2 production was obtained in the range of ~ 3 to 6 g L^{-1} glucose. Thus, the best medium composition for production of a new protein is determined as a compromise and might be used as a starting point for further optimization if repetitive or large scale production is envisioned. For further studies the combination of $\sim 3 \text{ g L}^{-1}$ glucose, $\sim 11 \text{ g L}^{-1}$ glycerol and $\sim 7.5 \text{ g L}^{-1}$ lactose was chosen and this medium named “Smart”-Defined Autoinduction Broth (S-DAB) (Table 3). It should be noticed, that this autoinduction medium is superior to conventional IPTG induction in defined medium regarding the amount of properly folded protein (see GST-GFP as an example in Figs. 1 and 2). The complete dataset showing the influence of carbon source combination on protein production using autoinduction are shown in Suppl. Figs. 1 and 2.

The influence of nitrogen content on protein production using autoinduction

The pH of the medium can be adjusted either by sodium or ammonium hydroxide, which increases either the salt (sodium) or the nitrogen content of the medium, respectively. When pH adjustment of S-DAB was carried out with ammonium instead of sodium hydroxide the ammonium content

increased from 60 to 120 mmol L⁻¹ decreasing the C/N ratio from 12 to 6 (C-mol/N-mol), respectively.

Adjusting the pH with ammonium instead of sodium hydroxide resulted in higher final cell densities and higher final volumetric product concentrations (Fig. 3). Moreover, for GFP and GST-GFP also a slight increase in the specific product concentration was observed while no changes were found for hFGF-2 (Suppl. Fig. 3). The percentage of soluble target protein was unaffected for GFP and hFGF-2, but for GST-GFP a substantial increase in inclusion body formation occurred when the medium pH was adjusted with ammonium hydroxide (Fig. 3). However, the volumetric concentration of correctly folded GST-GFP (also shown as GFP fluorescence per culture volume) was unaffected as the decreased solubility was compensated by the higher final volumetric GST-GFP concentration (Fig. 3). Thus, it is recommended to adjust the pH by using ammonium hydroxide. The corresponding medium has a Higher Nitrogen Content (HNC) and, thus, is named S-DAB (HNC) (Table 3).

The influence of oxygen supply on protein production using autoinduction

The oxygen supply in simple culture vessels can be improved by increasing the shaking speed and/or the culture surface to volume ratio. The analysis of oxygen supply on the production of GFP and hFGF-2 in S-DAB (HNC) clearly showed an increase in final cell density and volumetric product concentration for both proteins with increasing oxygen supply (Fig. 4). Moreover, better oxygenation also strongly decreased the formation of inclusion bodies during hFGF-2 production (Suppl. Fig. 4). Thus, protein production using autoinduction should be carried out under conditions which provide the best oxygenation. However, if sufficient oxygen cannot be supplied (e.g. growth in test tubes at 37°C) glycerol and lactose concentrations can be reduced (to ~7.5 and 4 g L⁻¹, respectively). The resulting Defined Autoinduction Broth (DAB) can be pH-adjusted with sodium hydroxide (Table 3).

Test of general applicability of S-DAB (HNC) for T7 based production systems

The general applicability of S-DAB (HNC) medium was tested for the production of 8 different proteins involving 3 different T7 based host strains (Table 1). Production using autoinduction in S-DAB (HNC) was compared to production in conventional LB medium using IPTG for induction of protein synthesis. This comparison revealed in general similar specific product concentrations using either S-DAB (HNC) or LB but final yields of target proteins were significantly higher in S-DAB (HNC) as about 4 times higher final biomass concentrations were reached (Fig. 5). However, it should be noted that the actual attainment is specific to the target protein. For example, in case of the protein Auto27-243-GST, the utilization of S-DAB (HNC) instead of LB not only resulted in 6 times higher cell densities but also significantly improved the specific product concentration and the solubility of the target protein. In most cases the improvement was less spectacular and in some cases a slight decline in specific product concentration or solubility was observed. Thus, S-DAB (HNC) can be used for the production of different types of proteins with usual excellent results, and, moreover, it can also be used for T7 based host strains which show poor growth characteristics such as *E. coli* Rosetta 2 (DE3).

Test of applicability of S-DAB (HNC) for screening studies in 96-well microtiter plates

The availability of a medium which omits inducer addition would greatly facilitate screening in 96-well microtiter plates for best production clones. Thus, the production of 8 different proteins involving 3 different T7 based host strains (Table 1) was tested in 96 deep well plates using S-DAB (HNC) (Fig. 6). In all cases the specific protein concentration was similar or even higher compared to production in shake flask cultures (refer to Fig 5). Moreover, in all cases higher final cell densities were obtained presumably as a result of evaporation. In summary it can be concluded that S-DAB (HNC) is very well suited for screening studies employing 96-well microtiter plates.

Test of applicability of S-DAB (HNC) for large scale cultivation

The applicability of S-DAB (HNC) for large scale cultivation was analyzed in 15-L bioreactors for the production of hFGF-2. Cultivations were carried out as pH-controlled batch process and also in the absence of pH control while keeping other culture variables identical. Both conditions were leading to nearly identical final cell densities and expression levels as were obtained in well-aerated shake flask cultures (Fig. 7 and refer to Fig. 5). The final pH in the non-pH controlled culture reached pH 5.9 indicating an excellent buffering capacity of S-DAB (HNC) and its applicability for large scale cultivation without pH control.

Discussion

A simple defined autoinduction medium was developed with general applicability for production of proteins with diverse properties using T7 based host strains with different growth characteristics. It can be employed in micro- as well as in macro-scale culture vessels leading to high final biomass concentrations ($OD_{600} > 20$) and average yields of 500 mg L^{-1} target protein. As there is no need for pH control and inducer addition, this defined medium represents a very convenient way for protein production. The final product levels are in average four times higher than can be reached in complex Luria Bertani broth through IPTG-induced protein production. The well-balanced composition of S-DAB (HNC) also becomes evident as the medium C/N ratio is close to the C/N ratio of the *E. coli* cell (Kayser et al. 2005) and the biomass yield coefficient close to the one at best attainable, e.g. 0.5 and 0.43 g g^{-1} for glucose and glycerol, respectively (Korz et al. 1995; Schmidt et al. 1999). Moreover, the defined composition only including basic compounds and no vitamin and amino acid supplements also renders it suitable for production of labeled proteins. However, special attention should be given to provide sufficient oxygen, e.g. growth at low temperature, high surface to volume ratio of culture broth. Under conditions of poor oxygen supply glycerol and lactose concentrations can be reduced.

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Figures captions

Fig. 1 Influence of temperature on protein production using autoinduction. Cells were grown in test tubes using autoinduction (T6, refer to Table 2) and production of GFP (□) and GST-GFP (■) followed using IPTG-induction in defined medium (DNB) as control. Upper panel: Relative fluorescence per nM of GFP (□) and GST-GFP (■). Final OD600 of GFP (□) and GST-GFP (■) producing culture. Lower panel: Volumetric concentration of GFP (□) or GST-GFP (■). Temperature and induction method are indicated below.

Fig. 2 Influence of carbon substrate composition on protein production using autoinduction. Cells were grown in test tube (narrow column) and shake flask (broad column). Upper panel: Fluorescence per 100 µl of GFP producing culture (□). Middle panel: Fluorescence per 100 µl of GST-GFP producing culture (■). Lower panel: Volumetric hFGF-2 concentration (▒). The light gray shadows indicate the maximum product yield area. Test tube and shake flask run numbers and glucose, glycerol and lactose concentrations are indicated below (refer to Table 2).

Fig. 3 The influence of nitrogen content on protein production using autoinduction. The nitrogen content of S-DAB was increased by using ammonium hydroxide [S-DAB (HNC)] for pH adjustment instead of sodium hydroxide (S-DAB). (A) Volumetric concentrations of GFP (□), GST-GFP (■), and hFGF-2 (▒) in S-DAB (left column) and S-DAB (HNC) (right column). (B) Fluorescence per 100 µl of GFP (□) and GST-GFP producing culture (■) in S-DAB (left column) and S-DAB (HNC) (right column). (C) Percentage of soluble (left column) and insoluble (right column) product amounts and final cell densities, respectively, of GFP (□, □), GST-GFP (■, ■), and hFGF-2 (▒, ▒) producing cultures in S-DAB and S-DAB (HNC).

Fig. 4 The influence of oxygen supply on protein production using autoinduction. Fluorescence per 100 µl of GFP producing culture (□) and volumetric hFGF-2 concentration (■) and final cell densities of the GFP (□) and hFGF-2 (■) producing culture using S-DAB (HNC). The experimental conditions were as follows: (A): 10 ml in 100 ml shake flask at 140 rpm; (B): 25 ml in 100 ml shake flask at 140 rpm; (C): 10 ml in 100 ml shake flask at 80 rpm; (D): 50 ml in 100 ml shake flask at 140 rpm; (E): 25 ml in 100 ml shake flask at 80 rpm; (F): 50 ml in 100 ml shake flask at 80 rpm.

Fig. 5 Test of general applicability of S-DAB (HNC) for T7 based production systems. Production of various proteins using different T7 based expression systems was carried out in Fernbach flasks using S-DAB (HNC) or IPTG-induction in Luria Bertani broth as control. Produced target proteins (refer to Table 1) and media are indicated above. Below are indicated the cell fraction analyzed by SDS-PAGE (W: whole cell protein. S: soluble part and I: insoluble part of whole cell protein) and the final cell densities (OD600).

Fig. 6 Test of applicability of S-DAB (HNC) for screening studies in 96-well microtiter plates. The following proteins (refer to Table 1) were produced in deep-well plates and the total cell protein analyzed by SDS-PAGE: hFGF-2 (lane 3), hPrP (lane 4), GFP (lane 5), PPAR γ (lane 6), co-production of Rab1A and SidM (lane 7), Auto27-243-GST (lane 8), GST-GFP (lane 9), and YopO-GST (lane 10). Protein standard (lanes 1 and 11) and plasmid free *E. coli* BL21 (DE3) are included as control (lane 2). The final cell densities are indicated below.

Fig. 7 Test of applicability of S-DAB (HNC) for large scale cultivation. hFGF-2 was produced in 15-L bioreactors (A) with pH control (pH 6.8) and (B) without pH control. All other culture variables were identical in both cultures. The concentrations of oxygen (—) and carbon dioxide (---) in the exhaust gas and the pH of the culture broth (·····) are shown. The inserts show SDS-PAGE analysis of whole cell protein from samples taken at indicated time points and final cell densities.

Tables

Table 1 Strains, plasmids and target proteins

Host strain	Plasmid	Target protein (MW)	Reference
<i>E. coli</i> BL21 (DE3)	pET-29c-hFGF-2	hFGF-2 (18 kDa)	(Hoffmann et al. 2004)
<i>E. coli</i> BL21 (DE3)	pET-28c-His6-GFP	GFP (27 kDa)	This work
<i>E. coli</i> BL21 (DE3)	pET-15b-hPrP	hPrP (29 kDa)	(Zahn et al. 1997)
<i>E. coli</i> BL21 (DE3)	pET-28a-PPAR γ	PPAR γ (33 kDa)	(Cronet et al. 2001)
<i>E. coli</i> Rosetta 2 (DE3)	PGEX-6P-1-Rab1A and pETM30-SidM	Rab1A (50 kDa) and SidM (53 kDa)	(Machner and Isberg 2006)
<i>E. coli</i> BL21 CodonPlus (DE3) RIL	pETM30- Auto27-243-GST	Auto27-243-GST (52 kDa)	(Bublitz et al. 2009)
<i>E. coli</i> BL21 (DE3)	pETM30-His6-GST-GFP	GST-GFP (54 kDa)	This work
<i>E. coli</i> BL21 (DE3)	PGEX-6P-1-YopO-GST	YopO-GST (90 kDa)	(Letzelter et al. 2006)

Table 2 Carbon source matrix for autoinduction medium development

Experiment test tubes	Experiment shake flasks	Glucose (g L ⁻¹)	Glycerol (g L ⁻¹)	Lactose (g L ⁻¹)	Carbon source (g L ⁻¹)
T1		0	0	3.8	3.8
T2		0	4.0	3.8	7.8
T3	S1	0	7.4	3.8	11.2
	S3	0	7.4	7.6	15.0
	S3	0	11.1	3.8	14.9
	S4	0	11.1	7.6	18.7
T4		2.9	0	3.8	6.7
T5		2.9	4.0	3.8	10.7
T6	S5	2.9	7.4	3.8	14.1
	S6	2.9	7.4	7.6	17.9
	S7	2.9	11.1	3.8	17.8
	S8	2.9	11.1	7.6	21.6
T7		5.9	0	3.8	9.7
T8		5.9	4.0	3.8	13.7
T9	S9	5.9	7.4	3.8	17.1
	S10	5.9	7.4	7.6	20.9
	S11	5.9	11.1	3.8	20.8
	S12	5.9	11.1	7.6	24.6
IPTG	control	10.9	0	0	10.9

Except for the concentration of carbon substrate(s), the concentrations of remaining components were identical and as follows: 4 g L⁻¹ (NH₄)₂HPO₄, 13.3 g L⁻¹ KH₂PO₄, 1.55 g L⁻¹ Citric acid, 0.59 g L⁻¹ MgSO₄, 100.8 mg L⁻¹ Fe(III) citrate, 2.1 mg L⁻¹ Na₂MoO₄·2H₂O, 2.5 mg L⁻¹ CoCl₂·6H₂O, 15 mg L⁻¹ MnCl₂·4H₂O, 1.5 mg L⁻¹ CuCl₂·2H₂O, 3 mg L⁻¹ H₃BO₃, 33.8 mg L⁻¹ Zn(CH₃COOH)₂·2H₂O, 14.1 mg L⁻¹ Titriplex III. The pH was adjusted with ~ 2.7 g L⁻¹ NaOH to pH 6.8.

Table 3 Optimized defined (autoinduction) medium

Medium	Application	Carbon source(s)	pH adjustment
S-DAB (HNC): “Smart”-Defined Autoinduction Broth (High Nitrogen Content)	Cultivation with good oxygen supply	2.9 g L ⁻¹ glucose 11.17 g L ⁻¹ glycerol 7.6 g L ⁻¹ lactose	about 4.5 ~5 ml L ⁻¹ NH ₃ .H ₂ O (~30 %) to reach pH 6.8
S-DAB: “Smart”-Defined Autoinduction Broth	Cultivation with intermediate oxygen supply	2.9 g L ⁻¹ glucose 11.17 g L ⁻¹ glycerol 7.6 g L ⁻¹ lactose	about 2.7 g L ⁻¹ NaOH to bring final pH to 6.8
DAB: Defined Autoinduction Broth	Cultivation with poor oxygen supply	2.9 g L ⁻¹ glucose 7.4 g L ⁻¹ glycerol 3.8 g L ⁻¹ lactose	
DNB: Defined Non- inducing Broth	IPTG induction or pre-culture	10.9 g L ⁻¹ glucose	

Except for the concentration of carbon substrate(s) and method of pH adjustment, the concentrations of remaining components were as in Table 2.

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6

Fig 7

Supplementary material

Suppl Fig. 1 Influence of carbon substrate composition on GFP production at 23°C using autoinduction.

Cells were grown in test tube (narrow column, small symbols) and shake flask cultures (broad column, big symbols). Upper panel: Expression level (○) and GFP fluorescence per 100 µl of GFP producing culture (□). Lower panel: Volumetric GFP concentration (□) and final cell density (□). The light gray shadow indicates the maximum product yield area. Test tube and shake flask run numbers and glucose, glycerol and lactose concentrations are indicated below (refer to Table 2).

Suppl Fig. 2 Influence of carbon substrate composition on GST-GFP production at 23°C using autoinduction.

Cells were grown in test tube (narrow column, small symbols) and shake flask (broad column, big symbols). Upper panel: Expression level (●) and GST-GFP fluorescence per 100 µl of GST-GFP producing culture (■). Lower panel: Volumetric GST-GFP concentration (■) and final cell density (■). The light gray shadow indicates the maximum product yield area. Test tube and shake flask run numbers and glucose, glycerol and lactose concentrations are indicated below (refer to Table 2).

Suppl. Fig. 3 The influence of nitrogen content on protein production and partitioning of the recombinant protein into soluble and insoluble cell fractions at 23°C using autoinduction.

Increasing the nitrogen content of S-DAB was accomplished by using ammonium hydroxide for pH adjustment instead of sodium hydroxide resulting in S-DAB (HNC). SDS-PAGE analysis of samples from duplicate experiments corresponding to whole cell protein, and soluble and insoluble fractions of cells producing (A) hFGF-2, (B) GFP and (C) GST-GFP, and. Arrows indicate the positions of the recombinant proteins. Below are indicated the cell fractions analyzed (W: whole cell protein. S: soluble part and I: insoluble part of whole cell protein) and above the medium employed.

Suppl. Fig. 4 The influence of oxygen supply on hFGF-2 production and partitioning of the recombinant protein into soluble and insoluble cell fractions at 23°C using S-DAB (HNC).

SDS-PAGE analysis of samples from duplicate experiments (X_1 and X_2) corresponding to whole cell protein, and soluble and insoluble cell fractions. Arrows indicate the position of hFGF-2. Below are indicated the cell fractions analyzed (W: whole cell protein. S: soluble part and I: insoluble part of whole cell protein) and above the experimental conditions employed (A): 10 ml in 100 ml shake flask at 140 rpm, (B): 25 ml in 100 ml shake flask at 140 rpm, (C): 10 ml in 100 ml shake flask at 80 rpm, (D): 50 ml in 100 ml shake flask at 140 rpm, and (E): 25 ml in 100 ml shake flask at 80 rpm.

Suppl. Table 1 Medium preparation

Groups		Name of medium	S-DAB	S-DAB (HNC)	DAB	DNB
		Application of the medium	Intermediate oxygen supply	Good oxygen supply	Poor oxygen supply	For IPTG induction or pre-culture
		Inducer	Lactose	Lactose	Lactose	IPTG
		Volume of stock solution	ml	ml	ml	ml
A group	Magnesium*	50 X MgSO ₄ stock solution	20	20	20	20
	Carbon source*	4 X 2.94-11.07-7.6 carbon source stock solution	250	250		
		5 X 2.94-7.38-3.8 carbon source stock solution			200	
		20 X Glucose stock solution				50
	Trace**	5000 times Na ₂ MoO ₄ .H ₂ O stock solution	0.2	0.2	0.2	0.2
B group	Elements**	2000 times trace element stock solution	0.5	0.5	0.5	0.5
	Nitrogen*	50 X (NH ₄) ₂ HPO ₄ stock solution	20	20	20	20
	Phosphate and other salts*	10 X KH ₂ PO ₄ stock solution	100	100	100	100
		50 X Citric acid stock solution	20	20	20	20
		50 X Fe(III) citrate stock solution	20	20	20	20
	pH adjusting*	25 X NH ₄ OH stock solution		45~50		
		50 X 5 mol L ⁻¹ NaOH stock solution	13.5		13.5	13.5
	Solvent†	Water (Milli-Q or similar quality)	556.5	520	606.5	756.5

A group: Mix magnesium and carbon source solution, sterilize. After adding 5000 X Na₂MoO₄.H₂O stock solution, the solution (A group) can be kept for long term storage at room temperature. **B group:** Mix nitrogen, phosphate and other salts and add solvent, adjust to pH 6.8, sterilize. After adding 2000 X trace element stock solution, the solution (B group) can be kept for long term storage at room temperature. * autoclave (or sterilize by filtration); ** sterilize by filtration.

Suppl. Table 2 Preparation of stock solutions

Name	Working concentration	Stock solution preparation	Sterilization method
50 X MgSO ₄ stock solution	0.586 g L ⁻¹ MgSO ₄	Add 30 g MgSO ₄ .7H ₂ O (MW: 246.47) to 500 ml graduated cylinder. Bring final volume to 0.5 L with water (Mill-Q or similar quality)	121°C autoclave or filtration (0.2 µm)
4 X 2.94-11.07-7.6 carbon source stock solution	2.94 g L ⁻¹ glucose 11.07 g L ⁻¹ glycerol 7.6 g L ⁻¹ lactose	Add 12.92 g glucose.H ₂ O (MW: 198.17), 44.28 g glycerol (MW: 92.09) and 32 g lactose.H ₂ O (MW: 360.31) to 1 L graduated cylinder. Bring final volume to 1 L with water (Mill-Q or similar quality)	121°C autoclave or filtration (0.2 µm)
5 X 2.94-7.38-3.8 carbon source stock solution	2.94 g L ⁻¹ glucose 7.38 g L ⁻¹ glycerol 3.8 g L ⁻¹ lactose	Add 16.15 g glucose.H ₂ O (MW: 198.17), 36.9 g glycerol (MW: 92.09) and 20 g lactose.H ₂ O (MW: 360.31) to 1 L graduated cylinder. Bring final volume to 1 L with water (Mill-Q or similar quality)	121°C autoclave or filtration (0.2 µm)
20 X Glucose stock solution	10.91 g L ⁻¹ glucose	Add 240 g glucose.H ₂ O (MW: 198.17) to 1 L graduated cylinder. Bring final volume to 1 L with water (Mill-Q or similar quality)	121°C autoclave or filtration (0.2 µm)
5000 X Na ₂ MoO ₄ .H ₂ O stock solution	2.1 mg L ⁻¹ Na ₂ MoO ₄ .2H ₂ O	Add 525 mg Na ₂ MoO ₄ .2H ₂ O (MW: 241.95) to 50 ml graduated cylinder. Bring final volume to 50 mL with water (Mill-Q or similar quality)	Filtration (0.2 µm)
2000 X trace element stock solution	2.5 mg L ⁻¹ CoCl ₂ .6H ₂ O 15 mg L ⁻¹ MnCl ₂ .4H ₂ O 1.5 mg L ⁻¹ CuCl ₂ .2H ₂ O 3 mg L ⁻¹ H ₃ BO ₃ 33.8 mg L ⁻¹ Zn(CH ₃ COO) ₂ .2H ₂ O 14.10 mg L ⁻¹ Titriplex III	Add 0.5 g CoCl ₂ .6H ₂ O (MW: 237.93), 3 g MnCl ₂ .4H ₂ O (MW: 197.9), 0.3 g CuCl ₂ .2H ₂ O (MW: 170.48), 0.6 g H ₃ BO ₃ (MW: 61.83), 6.76 g of Zn(CH ₃ COO) ₂ .2H ₂ O (MW: 219.49), 2.82 g Titriplex III (MW: 372.24) to 100 ml graduated cylinder. Bring final volume to 100 mL with water (Mill-Q or similar quality)	Filtration (0.2 µm)
50 X (NH ₄) ₂ HPO ₄ stock solution	4 g L ⁻¹ (NH ₄) ₂ HPO ₄	Add 100 g (NH ₄) ₂ HPO ₄ (MW: 132.06) to 500 ml graduated cylinder. Bring final volume to 500 mL with water (Mill-Q or similar quality)	121°C autoclave or filtration (0.2 µm)
10 X KH ₂ PO ₄ stock solution	13.3 g L ⁻¹ KH ₂ PO ₄	Add 133 g KH ₂ PO ₄ (MW: 136.09) to 1 L graduated cylinder. Bring final volume to 1 L with water (Mill-Q or similar quality)	121°C autoclave or filtration (0.2 µm)
50 X Citric acid stock solution	1.5542 g L ⁻¹ Citric acid	Add 42.5 g Citric acid.H ₂ O (MW: 210.14) [or 38.855 g Citric acid (MW: 192.12)] to 500 ml graduated cylinder. Bring final volume to 500 mL with water (Mill-Q or similar quality)	121°C autoclave or filtration (0.2 µm)
50 X Fe(III) citrate stock solution	0.1008 g L ⁻¹ Fe(III) citrate	Add 2.52 g Fe(III) citrate (MW: 244.94) to 500 ml graduated cylinder. Bring final volume to 500 mL with water (Mill-Q or similar quality)	121°C autoclave or filtration (0.2 µm)
25 X NH ₄ OH stock solution	To bring final pH to 6.8	Add 50 ml Ammonium hydroxide (30~33%) to 500 ml graduated cylinder. Bring final volume to 500 mL with water (Mill-Q or similar quality)	
5 mol L ⁻¹ NaOH stock solution	To bring final pH to 6.8	Add 100 g NaOH (MW: 40) to 500 ml graduated cylinder. Bring final volume to 500 mL with water (Mill-Q or similar quality)	