E. Coli Bioprocessing: Pioneering Advances in Biotechnology

Expert Insights

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Contents

Introduction

The field of *Escherichia coli (E. coli)* bioprocessing is of immense significance in the realm of biotechnology and pharmaceuticals. It enables production of vital substances, including life-saving drugs and proteins. The ability to manipulate and optimize *E. coli* processes can lead to more efficient production, addressing issues of availability and affordability of crucial drugs. Moreover, advancements in this field can enhance our understanding of biological processes, paving the way for innovative solutions in healthcare and beyond. This eBook aims to inspire and educate researchers about the potential of *E. coli* bioprocessing, particularly when leveraged with advanced bioreactor technology.

This Expert Insights begins with a study from Patil *et al*. on artemisinin production, a potent antimalarial drug, using a recombinant *E. coli* strain [1]. This research addresses the high price fluctuation and supply issues associated with artemisinin. By optimizing the biosynthesis process, it could improve the availability and affordability of this crucial drug, enhancing global malaria treatment efforts.

Next, Kumar *et al*. focused on the challenges and solutions in recombinant protein production (RPP) in *E. coli* [2]. The study is significant as RPP can cause amino acid depletion, leading to cellular stress. By supplementing limiting amino acids, they were able to enhance protein production and reduce cellular stress, thus improving the efficiency of RPP. This research could have broad implications for biotechnology and pharmaceutical industries.

Menacho-Melgar *et al*. investigated a novel strategy for the efficient expression of heterologous proteins in *E. coli*, a process crucial for many laboratories and industries [3]. Their method, activated by phosphate depletion and optimized across various scales and media, demonstrated broad applicability by successfully expressing ten diverse proteins. This research could significantly enhance protein engineering efforts and provide valuable insights into protein expression.

In addition to the article digests included in this collection, there is a whitepaper from IKA on recombinant protein production. This whitepaper emphasizes the advantages of bioreactor fermentation in comparison to shake flask procedures. The user-friendly nature of the HABITAT fermenter makes it an ideal choice when considering the transition from shake flask to bioreactor for your processes.

This collection explores the optimization of crucial biological processes in *E. coli*. It delves into the production of antimalarial drugs, addressing issues of availability and affordability. It also investigates the enhancement of recombinant protein production by managing amino acid depletion and cellular stress. Lastly, it presents a novel strategy for efficient heterologous protein expression. These themes collectively advance our understanding and application of biotechnological processes.

We hope to educate researchers on new technologies and techniques about *E. coli* bioprocessing, through the methods and applications presented in this Expert Insights. To better understand the available options for improving your research with IKA's HABITAT bioreactors, we encourage you to visit their website.

Christene A. Smith, PhD
Editor at Wiley

References

[1] Patil, V. *et al*. (2021). Balancing glucose and oxygen uptake rates to enable high amorpha-4,11-diene production in Escherichia coli via the methylerythritol phosphate pathway. *Biotechnology and Bioengineering*. DOI: 10.1002/bit.27655.

[2] Kumar, J. *et al*. (2020). Amino acid supplementation for enhancing recombinant protein production in E. coli. *Biotechnology and Bioengineering*. DOI: 10.1002/bit.27371.

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Balancing Glucose and Oxygen Uptake Rates to Enable High Amorpha-4,11-Diene Production in *Escherichia coli* via the Methylerythritol Phosphate Pathway

Adapted from Patil *et al.*, 2020

Artemisinin is a potent antimalarial drug extracted from *Artemisia annua* shrubs. The World Health Organization (WHO) recommends its use in combination with conventional drug therapies to slow the development of resistance in the malarial parasite. Despite numerous efforts to use biotechnology to efficiently produce one of the precursors of artemisinin, more efficient biosynthetic strategies are needed due to the high price fluctuation and supply issues associated with it.

The strategy herein is to optimize key process conditions for synthesizing artemisinin sesquiterpene precursor amorpha-4,11-diene (AMD4,11) by a recombinant *Escherichia coli* strain using its endogenous methylerythritol phosphate (MEP) pathway in 2L bioreactors. Elucidation of the interplay between oxygen demand and glucose uptake rates enabled the manipulation of NADPH availability and optimization of carbon flux through the MEP pathway. Product titers increased 6.7-fold after the installation of optimized oxygen and glucose ($q_{O2}/q_{glucose}$) conditions.

Introduction

WHO reported that malaria caused approx. 405,000 deaths in 2018, mainly from treatment-resistant *Plasmodium sp*. infections. The WHO began recommending that malarial treatment include the herbal preparation of artemisinin in combination with conventional therapies, in hopes of slowing the generation or spread of resistant *Plasmodium sp*. Artemisinin is extracted from the *Artemisia annua* shrub and its price can vary up to 10-fold. Several groups have pursued the production of one of its precursors by biosynthetic routes, but processes have not yet met their economic targets.

The sesquiterpene lactone peroxide artemisinin can be produced from its sesquiterpene precursor, amorpha-4,11-diene (AMD4,11) by photochemical conversion. AMD4,11 can be enzymatically produced from farnesyl pyrophosphate (FPP) which can be formed from two common precursors terpenoids; isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Both IPP and DMAPP can be generated by the methylerythritol phosphate (MEP) pathway in bacteria, such as *E. coli*. The advantages of using the MEP pathway for terpenoid production include its high theoretical yield of carbon and the relatively short duration of *E. coli* fermentation batches. Patil *et al*. investigated the effects of manipulating several key parameters. They controlled the relative oxygen and carbon rates during the 2L bioreactor run, meeting essential energy and cofactor demands, and achieved sustained higher productivity (carbon yield >5% and biomass >1.8 mg/dry-cell-weight-hour (DCW-h)).

Comparison of Fermentation Conditions

Comparison of 2L bioreactor runs between a monophasic system or a biphasic system (10% oil) initially revealed that at 71 hours, the monophasic system yielded higher peak biomass concentrations (26 gDCW/L) than the biphasic system (18 gDCW/L). Similarly, at 71 hours, the monophasic system produced more than fivefold higher AMD4,11 (3.5 g/L) than the biphasic system (0.6 g/L). However, time course studies revealed that the biphasic system had initially much higher AMD4,11 production (5.5 mg/DCW/h), which then declined. Cost-effective IKA bioreactors and fermenters of various sizes can be assembled to provide multiple monitoring channels to improve efficiency. Additional time course studies monitored the intracellular concentrations of ATP and two metabolites of the MEP pathway (1-deoxy-D-xylulose (DOX), and (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (MEcPP)) to assess its functionality and flux. The peak concentrations and time courses of ATP, DOX, and MEcPP differed greatly between the monophasic and biphasic systems. The accumulation of DOX (up to 10-fold) in

the biphasic system raised the possibility that sufficient NADPH was not available for its cofactor role in the MEP pathway, and NADPH could become a limiting factor in the AMD4,11 production by the MEP pathway.

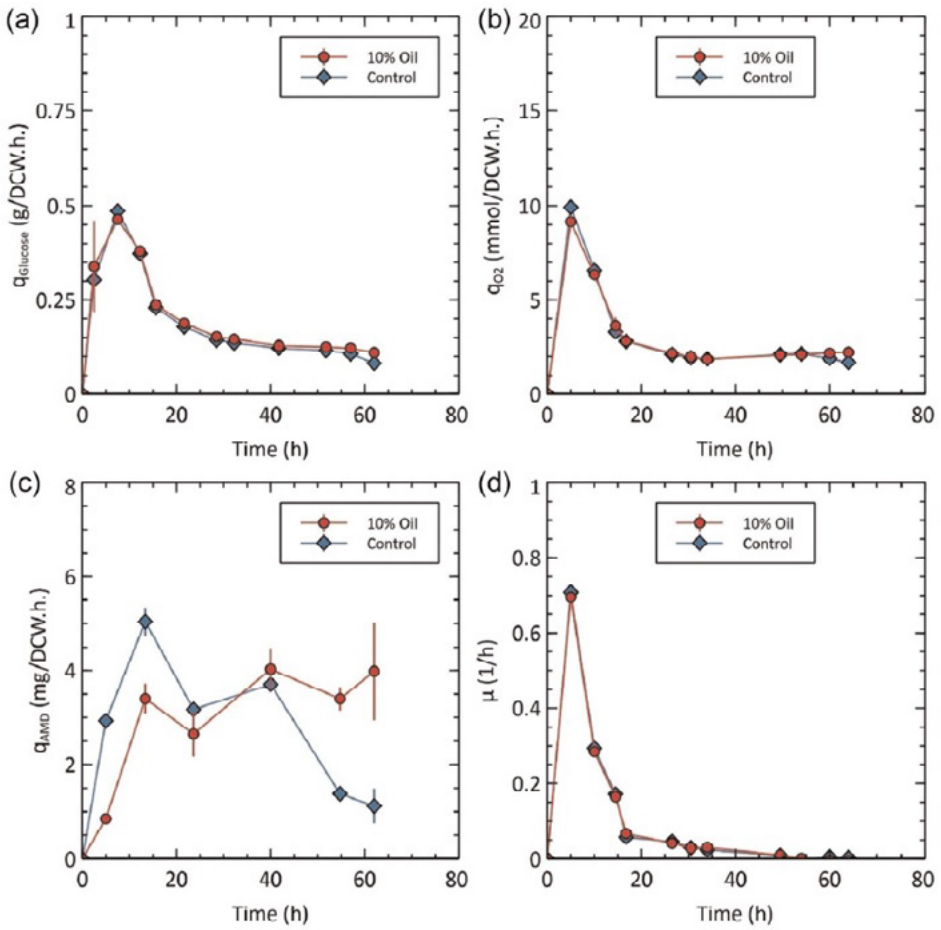
Flux Balance Analysis

Flux balance analysis (FBA) implied that the carbon flux distributions in the two systems were similar although the activity of the MEP and the citric acid (TCA) pathways showed blatant differences. Patil *et al*. used the differences in the calculated fluxes for NADPH formation, NAD transhydrogenase, and ATP synthesis reactions of the monophasic and biphasic bioreactor runs to hypothesize that a high $q_{O2}/q_{glucose}$ ratio may reduce the functionality of the MEP pathway by restricting the availability of two key substrates (NADPH and pyruvate).

Optimizing Glucose and Oxygen Uptake Rates to Improve Performance of Biphasic System

The oil overlay in the series 1 biphasic bioreactor runs had reduced the aqueous volume which slightly increased the glucose concentrations. In series 2 experiments, Patil *et al*. balanced the oxygen uptake and glucose uptake rates in the biphasic system by reducing the glucose input rate by 10% to compensate for the oil overlay-induced volume reduction. The same aeration procedure was used. The series 2 bioreactor monophasic and biphasic system runs showed similar levels of glucose uptake rates, oxygen uptake rates, and biomass production throughout the time course (Fig. 1a, b, d). For most of the fermentation, the biphasic runs maintained a cell-specific AMD4,11 productivity of approx. 4 mg/DCW/h, at least

Figure 1



Biomass-specific dynamics for series 2 runs of monophasic system (control) and biphasic system (10% oil) in 2L bioreactors. Time course of (a) glucose-specific uptake rate ($q_{glucose}$) in g/DCW-h; (b) oxygen-specific uptake rate (q_{O2}) in mm/DCW-h; (c) specific productivity of AMD4,11 (q_{AMD}) in mg/DCW-h; and (d) growth rate (μ) in 1/h. DCW, dry cell weight.

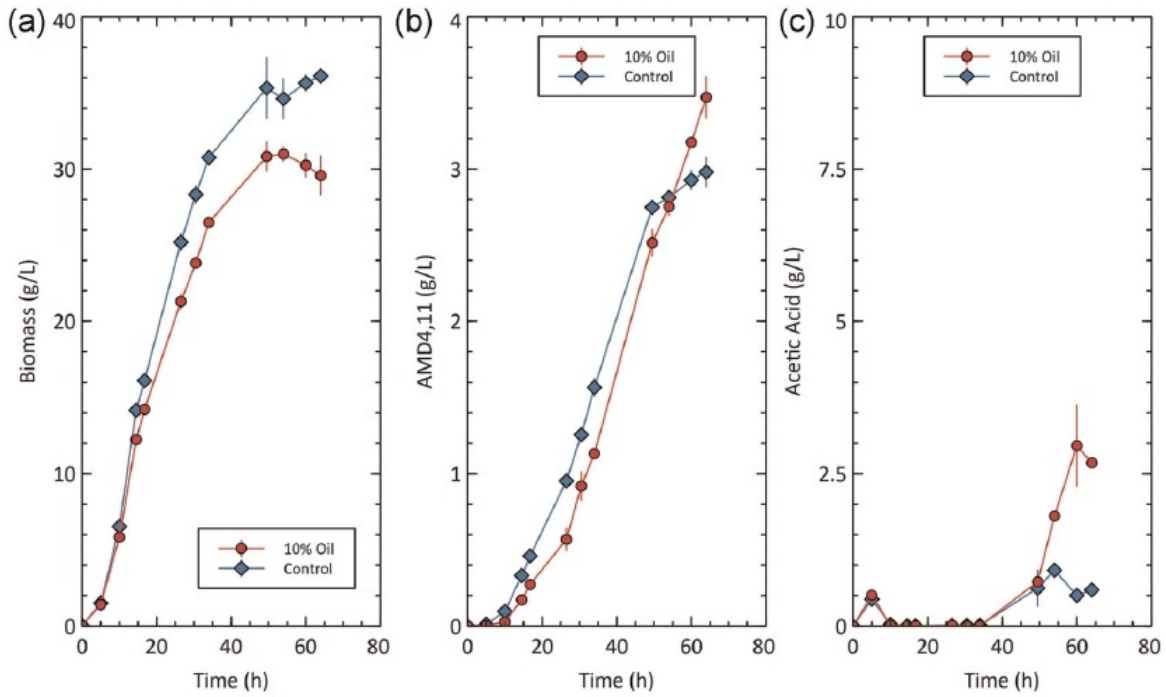
matching the AMD4,11 productivity of the monophasic system (Fig. 1c). The two conditions also induced similar profiles of intracellular concentrations of ATP, DOX, and MEcPP throughout the runs. Interestingly, the intracellular levels of DOX and MEcPP steadily climbed during the fermentation. The increased availability of MEcPP for use in the MEP pathway suggests higher AMD4,11 production.

Both monophasic and biphasic series 2 bioreactor fermentations showed similar levels of accumulation of AMD4,11 protein, with the biphasic system possibly exceeding production of AMD4,11 at the last two time points (Fig. 2b). The series 2 biphasic conditions increased the AMD4,11 titers by six-fold and the conversion yield by almost seven-fold. The other parameters (calculated and measured) of both series 2 monophasic and biphasic fermentations displayed similar trends. These results indicate that the balancing of the $q_{O2}/q_{glucose}$ ratio improved pyruvate and NADPH availability and enabled higher MEP pathway activity which led to AMD4,11 production.

Summary

Patil *et al*. established that an oil overlay modestly reduces the volume in a 2L bioreactor, but that modest volume reduction can alter the glucose concentrations sufficiently to negatively impact the production of heterologous proteins in *E. coli* fermentations. Time course studies combined with monitoring members of the MEP pathway and flux balance analysis revealed that a higher $q_{O2}/q_{glucose}$ ratio moderates the MEP pathway flux and reduces the production of AMD4,11. Reduction of glucose feed rates by 10% in the biphasic fermentations rebalanced the $q_{O2}/q_{glucose}$ ratio and improved AMD4,11 production. Comparisons of the yields of the two fermentation conditions indicate the importance of investigating the reasons for lower production to elucidate limiting factors and help design adjustments to match or exceed the production of the heterologous protein in *E. coli* inoculated bioreactors.

Figure 2



Key output characteristics of the series 2 bioreactor fermentations under the monophasic (control) and biphasic (10% oil overlay with 10% reduction of glucose input rate) conditions. (a) biomass concentration (g/L), (b) AMD4,11 titer (g/L), and acetic acid concentration (g/L).

Amino Acid Supplementation for Enhancing Recombinant Protein Production in *E. coli*

Adapted from Kumar *et al.*, 2020

Recombinant protein production (RPP) in *Escherichia coli* redirects the consumption of amino acids from normal metabolic processes and can compromise cellular growth. RPP can cause amino acid starvation and metabolic stress, leading to activation of the stringent-like response, a global stress response. The consumption profile indicated that several amino acids (AAs) stores were exhausted or depleted during cellular growth (GP1) and/or protein production (GP2). Cellular growth and production of the recombinant protein pramlintide were enhanced with amino acid supplementation in a chemically defined medium (CDM). Supplementation with GP1 and GP2 AAs increased pramlintide production by 40% and concurrently decreased the expression of genes associated with AA synthesis and global stress response. Thus, supplementation of limiting AAs increased recombinant protein production and decreased cellular stress during RPP.

Introduction

Production of recombinant proteins in *Escherichia coli (E. coli)* accounts for ≈31% of approved therapeutic proteins in European and US markets. Challenges with RPP in *E. coli* include low protein activity, insoluble aggregates, metabolic stress, and reduced cell growth. Metabolic stress due to AA starvation stimulates the stringent control network which disrupts stable RNA complexes and protein synthesis, stimulates AA synthesis and its transport, activates multiple stress genes, regulates nutritional stress responses, and promotes biofilm formation.

Several previous studies showed that specific AA supplementation in a chemically defined medium (CDM) increased the production of heterologous proteins. Herein, Kumar *et al*. describe their discovery and characterization processes to elucidate the specific AAs needed for supplementation to boost the production of their specific protein, recombinant pramlintide (rPramlintide). Scale-up of RPP in bioreactors was characterized for cell growth and RPP.

Elucidation Of Limiting Amino Acids

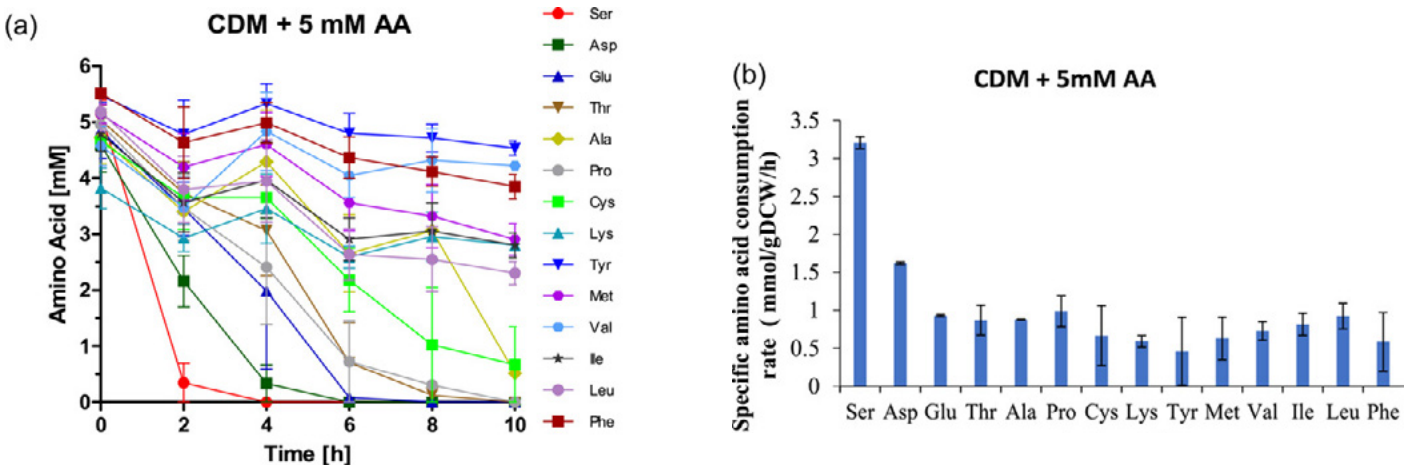
The preliminary research to elucidate which AAs became limiting during the cell growth stage of *E. coli* (up to 6 h) and the RPP stage (6-10 h) was performed in flasks using AA-supplemented CDM. rPramlintide production modestly increased with 2.5 mM AAs and significantly increased with both 5 mM AAs and 7.5 mM AA supplementation. Because both 5 mM and 7.5 mM AA supplementation yielded similar levels of RPP, subsequent experiments were performed with 5 mM AAs.

Consumption profiles in AA-supplemented flask cultures revealed that serine (SER), aspartic acid (ASP), and glutamic acid (GLU) were completely depleted by 6 h, i.e., before rPramlintide production (Fig. 1a). During the initial 6 h of culture, proline (PRO) and threonine (THR) were also consumed more rapidly than the remaining AAs. During the 6-10 h post inoculation (protein production stage), the cells showed faster uptake of alanine (ALA), cysteine (CYS), leucine (LEU), and methionine (MET) from the AA-supplemented flask cultures (Fig. 1a).

AA Supplemental Design

Kumar *et al*. sought an economically viable AA supplementation for RPP in *E. coli* cultures, before switching to bioreactors. Because supplementation with all 20 AAs is too expensive, they compared the no supplementation control (SF1) and all-AA mix (SF6) against four different AA supplementation combinations (Table 1). The AAs in GP1 promoted growth and included aspartic acid, glutamic acid, proline, serine,

Figure 1



Consumption profiles of amino acids and consumption rates in shake flask cultures of *E. coli* that are engineered to express recombinant protein rPramlintide. Chemically defined medium (CDM) was supplemented with 5 mM AAs. (a) Consumption profiles of the 14 AAs as a function of time (2-10 h). (b) Consumption rates of 14 individual AAs were calculated at 2 hours, during the initial exponential growth phase. AAs, amino acids; CDM, chemically defined medium; DCW, dry cell weight; the three-letter abbreviations are used for individual amino acids

and threonine. The AAs in GP2 enhanced rPramlintide production and included alanine, cysteine, leucine, and methionine. Glucose consumption profiles and cell density of the AA-supplemented flasks were not significantly different from the no-supplementation control flasks (SF1). In contrast, the AA-supplemented flasks yielded significantly higher protein yields. The SF5 flasks reached a maximum product yield of 110.3 ± 9.54 mg rPramlintide per gDCW and rPramlintide concentration of 0.47 ± 0.04 g/L (data not shown).

Table 1 shows that supplementation with GP1 alone (SF2), or both GP1 and GP2 AAs yielded high rPramlintide production (SF3, SF5). The SF5 results show that GP1 supplementation at inoculation and both GP1 and GP2 supplementation at the time of induction yielded the highest rPramlintide production of flasks receiving economically viable AA supplementation. Analogously, the SF5 conditions also increased the production of granulocyte-colony stimulating factor (GCSF) protein production by recombinant *E. coli* strain with GCSF-expressing plasmid.

Scale-Up Bioreactor Run

The SF5 supplementation strategy was then compared to a control in 1.3 L bioreactors. The control and supplemented test runs showed maximum specific growth rates during batch phases of 0.56 ± 0.07 and 0.59 ± 0.03 per hr. The end of the batch phase in both control and test runs displayed a sharp rise in dissolved oxygen (Fig. 2 a-d). In all runs, RPP was induced by

Table 1 μ_{max}, specific growth rate; Y_{P/S_glu}, product yield coefficient on glucose.

Shake flask	Inoculation (Hour 0) 5 mM GP1	Induction (hour 6) 2.5 mM GP1	Induction (hour 6) 2.5 mM GP2	μ_{max} / hr	Y_{P/S_glu}, (mg protein/ glucose)
SF1	-	-	-	0.32 ± 0.1	8.73 ± 0.56
SF2	+	-	-	0.42 ± 0.08	26.70 ± 2.54
SF3	+	-	+	0.45	35.93 ± 3.95
SF4	-	-	+	0.43 ± 0.07	13.73 ± 2.36
SF5	+	+	+	0.33 ± 0.06	45.74 ± 3.56
SF6	5 mM all AAs	-	-	0.48 ± 0.09	71.30 ± 9.24

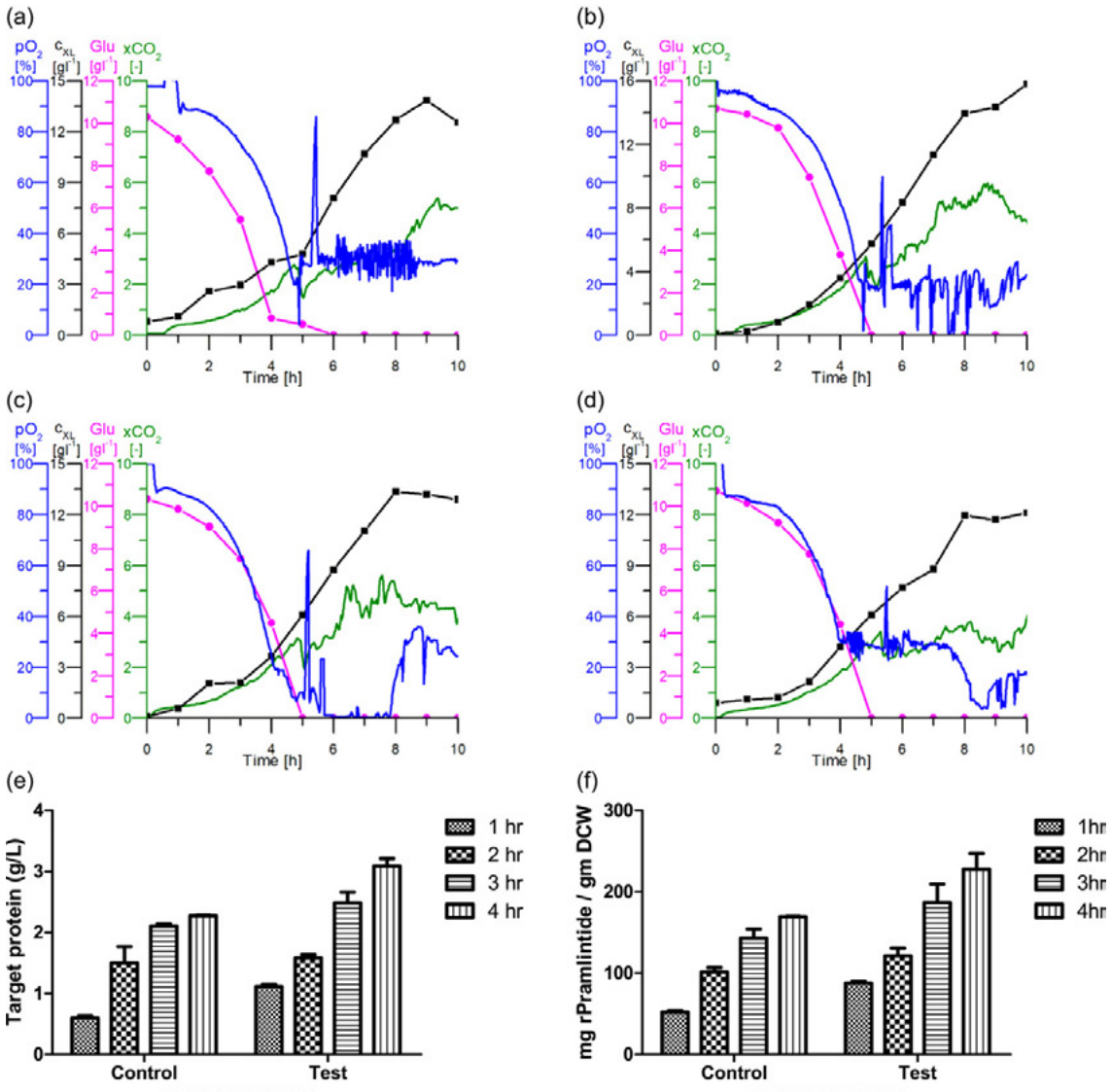
Growth rate and rPramlintide production in GP1 and GP2 supplementation study in shake flasks

adding isopropyl β-d-1-thiogalactopyranoside (IPTG) at 6 h. Hourly monitoring revealed a steady increase in rPramlintide production for the full four hours in the test runs but a slow-down in protein production after the second hour in control runs. The rPramlintide multimer concentration obtained in the test bioreactor was 40% higher (3.09 ± 0.12 g/L) than that obtained in the no AA supplementation control bioreactor (2.27 ± 0.01 g/L) (Fig. 2e). Similarly, the maximum protein yield of the test bioreactors was higher (227.69 ± 19.71 mg rPramlintide multimer per gDCW) than that of the control bioreactors (169.39 ± 0.51 mg/gDCW). IKA bioreactors and fermenters provide automated sensors for turbidity, CO_2, and conductivity to improve efficiency.

Summary

Kumar *et al*. investigated the potential role of RPP-induced metabolic burden on the overall growth and productivity in recombinant *E. coli* cultures. Determination of the uptake profile of amino acids revealed a set of AA depleted or predominantly consumed during the growth phase (GP1) and a second set of AAs consumed rapidly during protein production (GP2). Bioreactor runs supplemented with both G1 and GP2 produced approx. 40% higher rPramlintide yields (3.1 g/L). These data support the elucidation of mechanisms underlying metabolic burden and implementing a strategy that alleviates the stress and improves RPP production yields.

Figure 2



Monitored characteristics of fed-batch bioreactor runs. (a, b) no AA supplementation control bioreactor runs. (c, d) Test bioreactor runs using SF5-based supplementation of GP1 and GP2. (a) Dissolved oxygen (pO_2) profile; (b) cell density (C_{XL}) profile; (c) Glucose consumption (Glu) profile; (d) exhaust CO_2 (xCO_2) profile. (e) concentration of target protein (g/L): postinduction profile of recombinant pramlintide (rPramlintide g/L) in control and test runs at indicated times. (f) Target protein yield: quantity of rPramlintide produced (g/DCW) after 1 to 4 hours postinduction in control and test runs. DCW, dry cell weight.

Scalable, Two-Stage, Autoinduction of Recombinant Protein Expression in *E. coli* Utilizing Phosphate Depletion

Menacho-Melgar *et al.*, 2020

Many laboratories and industries rely on the efficient expression of heterologous proteins in *Escherichia coli* to elucidate novel insights and engineer improved proteins. The design, engineering, and characterization of a novel strategy for the expression of recombinant proteins using a promoter activated by phosphate depletion is described. This method employs tightly controlled autoinduction and can be optimized in rich media and chemically defined media at scales ranging from 20 µL to 100 mL and fed-batch fermentations. This strategy can be optimized to yield heterologous proteins as high as 55% of total cell protein. The approach has been used to express ten diverse heterologous proteins, demonstrating its broad applicability.

Introduction

Recombinant *Escherichia coli (E. coli)* is often used to express heterologous proteins for research, industrial, and healthcare purposes. Most *E. coli*-based expression systems optimize protein expression during the exponential growth phase. Various inducible promoters such as IPTG (isopropyl β-D-1 thiogalactopyranoside) are currently used, and each expression cassette has its advantages and challenges for expressing specific proteins. Scale-up of protein expression in flasks and bioreactors can be complicated by the accumulation of acetic acid, heterologous gene-induced stress, or leaky induction. Ideally, a facile reproducible *E. coli*-based strategy would include the following characteristics: tightly controlled protein expression that can be automatically induced, high yields of recombinant protein, and minimal overflow metabolism.

Menacho-Melgar *et al.*, describe the development of a reproducible strategy in a modified distinct *E. coli* strain (BW25113) that yields high protein expression during the stationary phase. Stationary phase cells continue metabolic activity while expressing high protein levels. Several phosphate-modulated promoters in recombinant *E. coli* strains can tightly control and amplify heterologous protein expression with minimal acetate production. Cultures can be managed so phosphate depletion occurs at the beginning of the stationary phase. Instead of requiring rich media for growth, some of these strains can be cultured in minimal media or autoinduction broth in multiple vessels, including controlled batch-fed bioreactors to 384 plates with 20 µL.

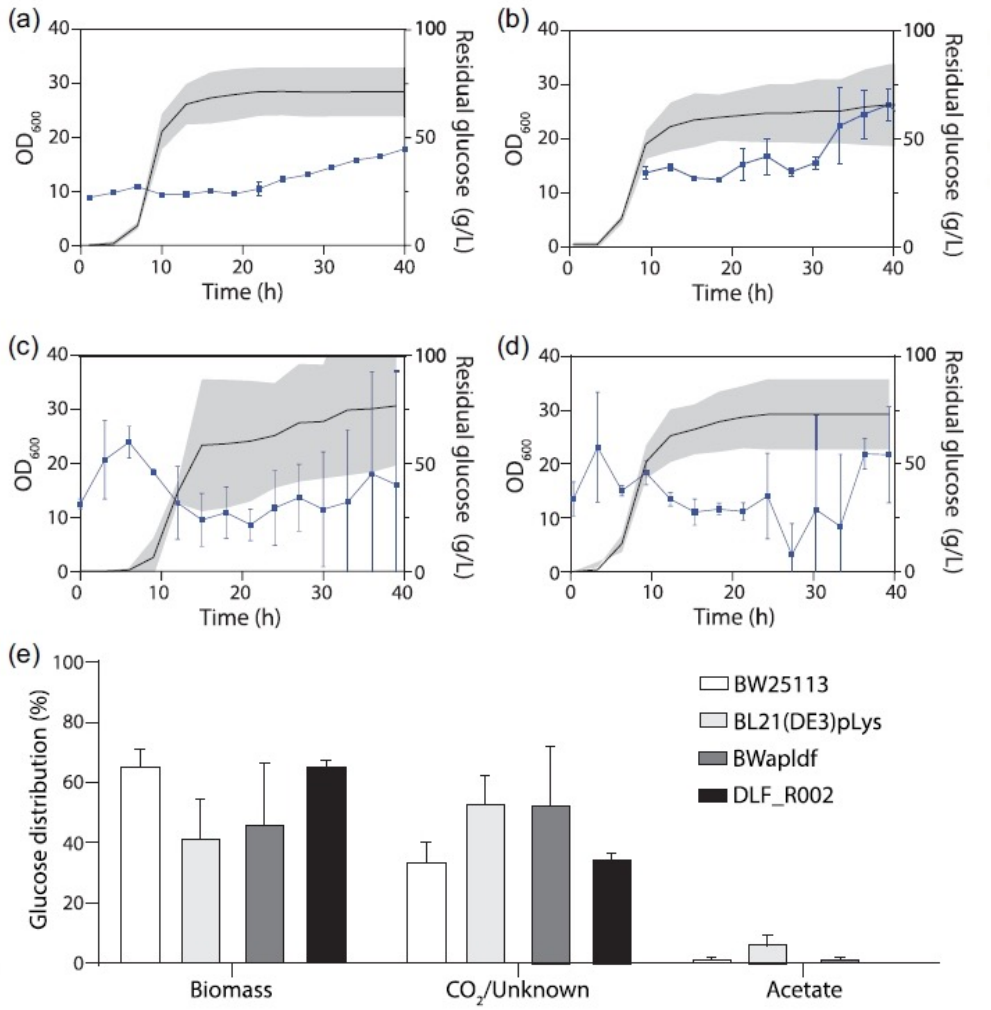
Characterization of yibDp Promoter Activity

To improve the inducibility and yields of heterologous proteins in stationary *E. coli* cultures in bioreactors, the authors chose to engineer a modified phosB-regulated promoter of the yibD gene (yibDp promoter) which is activated by phosphate depletion. Initially, the activity of the yibDp promoter was monitored with the mCherry reporter gene in three *E. coli* strains: an *E. coli* K-12 derivative BW25113, the relatively common BL21(DE3), and BL21(DE3) with the pLysS accessory plasmid that previously has reduced leaky expression. The yibDp coupled with the mCherry reporter showed basal promoter activity in the commonly used *E. coli* strain BL21(DE3). The yibDp promoter showed no basal expression in *E. coli* strain BW25113. The mechanisms for basal expression were not pursued. Subsequent experiments with this yibDp promoter were performed in BW25113 or one of its derivatives such as BWapldf which was designed to produce fewer overflow metabolites (e.g., acetate).

Growth Characteristics of Four *E. Coli* Strains

The 1L bioreactor fed-batch fermentations in minimal media (FGM10) compared the biomass, growth rates, and formation of byproducts such as acetic acid in four *E. coli* strains of potential interest for heterologous protein production (Fig. 1). These fermentations did not include optimization of feed rates, and probably

Figure 1



Growth and acetate production of four *E. coli* strains fermented in minimal media (FGM10) in 1 L bioreactors. Results represent averages of duplicate fermentations. (a-d) Biomass levels (black line) and residual glucose concentrations (blue line) for (a) BL21(DE3)pLys, (b) BW25113, (c) BWapldf, and (d) DLF_R002. (e) Glucose distribution utilized by the four strains during fermentation in minimal media. CO_2 levels were measured via off-gas analysis in BW25113, BWapldf, and DLF_R002, but considered an unknown product for BL21(DE3)pLys.

included excess residual glucose. The growth rates of strains BL21(DE3)pLys, BW25113, and DLF_R002 were similar whereas BWapldf appeared to have a longer lag period. Interestingly, the DLF_R002 fermentation, despite residual sugar, showed no detectable acetate.

To evaluate yields of heterologous protein production in bioreactor fermentations, the authors switched the reporter gene to the green fluorescent protein by using their engineered plasmid, pHCKan-yibDp-GFPuv. Fermentations in FGM10 media in *E. coli* are limited by the low phosphate levels. When biomass levels reach approx. 10 gCDW/L or 30-35 OD (low cell density) in *E. coli* strains DLF_R002 or DLF_003, the depleted phosphate levels induced the expression of green fluorescent protein (GFP) from the pHCKan-yibDp-GFPuv plasmid. These biomass levels of 10 gCDW/L were maintained for at least 20 – 30 h. The fermentations in FGM10 yielded GFP production of approx. 2.7 g/L or 270 mg/gCDW (Fig. 2a).

Menacho-Melgar *et al*. tested whether 1L bioreactor fermentations in a richer media (FGM30) would not only support higher cell densities but also produce higher titers of GFPuv. As shown in Figure 2b, 1 L bioreactor fermentations in *E. coli* strain DLF_R003 with FGM30 media supported higher optical densities (approx. 75-80) and induced about a three-fold higher production of GFPuv titer (approx. 8.1 g/L). Taken together, these 1 L fermentation results support the continued development of the yibDb promoter-driven expression system in several *E. coli* strains. These bioreactor runs can be performed in IKA fermenters and/or photoreactors with automated sampling, autoinduction, and ergonomic features.

Optimization and Broad Applicability

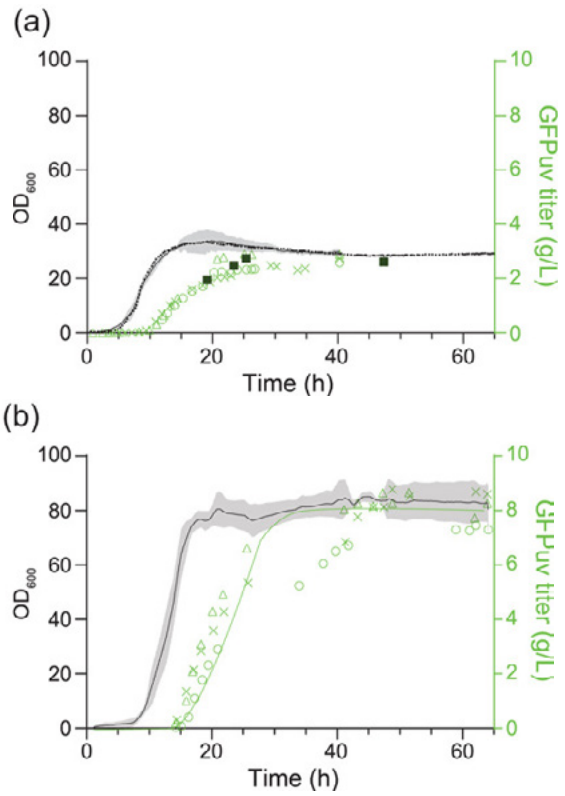
Further refinement of the media components to support the growth and stationary phase of DLF_R002 with plasmid pHCKan-yibDp-GFPuv and to amplify the GFPuv protein production used a design of experiment (DOE) methodology in 96-well plates. Of the tested 212 media formulations, the media formulation named AB enabled autoinduction of the DLF_R002 *E. coli* strain with pHCKan-yibDp-GFPuv plasmid, resulting in high expression of GFPuv. Secondly, optimization of autoinduction of pHCKan-yibDp-GFPuv in DLF_R002 revealed that higher volumes of media in 384- or 96-well plates and the flasks produced lower levels of GFPuv (g/L).

Autoinduction of *E. coli* strain with a plasmid containing the yibDp promoter driving expression of one of eight additional distinct heterologous proteins yielded the heterologous protein accounting for approx. 10% to 55% of total protein, demonstrating the broad applicability of this construct.

Summary

The yibDp plasmid in the DLF_R002 *E. coli* strain supports autoinduction, no basal expression, high heterologous protein expression during a long stationary phase, and minimal production of acetate. Another major advantage of the yibDp system is the ability to develop high throughput screens in 384-well plates and use the same plasmid-*E. coli* strain combination for scale-up of heterologous protein production in bioreactors. Using IKA bioreactors or fermenters with photosensors and monitoring channels can greatly enhance the efficiency of optimizing production conditions and performing production runs.

Figure 2



Comparison of autoinduction of Green Fluorescent Protein (GFPuv) driven by yibDp promoter (plasmid HCKan-yibDp-GFPuv) in 1 L bioreactors using two different types of minimal medium and two different hosts. (a) FGM10 minimal media and host strain DLF_R002 runs were performed in triplicate (solid line, open circles) whereas run with DLF_R003 host strain was performed once (dotted line, filled squares); (b) FGM30 minimal media and host strain DLF_R003. Optical density (black lines) and normalized GFPuv fluorescent units (green X's, triangles, circles, or squares) were measured over time. Standard error of triplicate growth profiles represented by the shaded area. Best fit of expression profiles represented by a line. OD, optical density.

Recombinant Protein Production - The Advantages of Fermentation in Bioreactors (Fermenters) Compared to Shake Flask Cultures

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May 2024, IKA-Werke GmbH & Co. KG, Staufen, Germany

Abbreviations: *E. coli, Escherichia coli*; DO, Dissolved oxygen; ID, Inner diameter

Introduction – Recombinant Protein Synthesis

Recombinant protein synthesis is a major advancement made in biotechnological applications. The process effectively utilizes the cell's machinery to manufacture substances of exceptional complexity and specificity [1].

This paper focuses on the use of bioreactors to produce recombinant proteins. Bioreactors are vessels that can serve as fermenters, providing an optimal environment for microbial cells to grow and produce the desired protein. Depending on the complexity of the protein, either bacterial cells (such as *E. coli*) or mammalian/insect cells are used in these bioreactors (fermenters) to achieve the desired outcome.

The recombinant proteins are encoded by recombinant DNA that are cloned into an expression vector. They are produced through cellular protein synthesis that transforms bacteria with the expression vector containing the recombinant DNA. Amino acid exchanges, truncations, or fusion proteins are generated by modifying the gene through recombinant DNA technology.

Recombinant proteins often serve important roles in industrial applications or are used in diagnostic tests or therapeutic purposes. A previous whitepaper by IKA on plasmid DNA transformation in *E. coli* highlighted considerations for effective techniques [2].

The selection of the right expression system is important [3]. Factors to consider when choosing protein expression systems include structural complexity, post-translational modifications, and solubility of the expressed protein. The structural complexity of a protein may indicate which system is most suitable for recombinant generation. If budget constraints are a concern, bacteria, plant cells, or fungi are typically more cost-effective. However, the complexity of the protein's post-translational modifications, especially glycosylation and proper folding, is important.

E. coli is the most frequently used host for production of enzymes and other proteins by recombinant DNA technology [4]. *E. coli* is preferable due to its relative simplicity, inexpensiveness, well-known genetics, and compatibility with a large number of molecular tools. Bacterial protein expression systems produce high yields of recombinant protein in very short time frames. Antibiotics can be added to prevent contamination by cells other than the protein-producing host cells. Multiple bacterial strains and cell lines are available for protein expression.

Cultivation Practices Shifting to Bioreactors (Fermenters)

For years, shake flasks have been the "go-to" molecular tool to cultivate bacteria, plant and fungi cells in suspension. While inexpensive and easy to use due to lack of sophisticated instrumentation, they are best suited for the screening process and small-scale production in the early stages.

Looking for increased capacity to produce greater yields at higher cell densities, the industry is shifting its cultivation practices towards the use of fermenters. In addition to providing greater control over the cellular environment than traditional bacterial cultivation methods such as a flask-based culture, this disruptive technology offers predictable scalability to move to commercial levels.

A fermenter provides a closed system in which all parameters for optimal growth are constantly monitored and recorded. In addition to avoiding potential human errors, processes developed in a fermenter with sophisticated sensor technologies are more reproducible than shake flask experiments. Using recorded data, the evaluation of fermentation runs and creation of meaningful graphs for scientific publication are very easy.

In this publication, we will explain the challenges associated with shake flasks and how an automated bioreactor (fermenter) can overcome these by adopting suitable bioprocessing strategies.

The Limitations of Shake Flask Cultures

The shake flask culture is a well-established laboratory method for culturing bacteria and other microorganisms. Because of their utility, flexibility and low cost, shake flasks are popular for bacterial growth in academic research and bioprocess engineering. Often used for screening and process development, scale-up is possible but requires investing in numerous flasks, which in turn demands more lab capacity.

In the shake flask culture, bacteria are grown in Erlenmeyer flasks that are limited to volumes up to 5 litres. The shape of the flasks allows for efficient shaking performance without spilling the liquid. Modified flasks can provide an optimized surface-to-volume ratio while baffled designs can enhance aeration. Different flask closures prevent contamination and allow for gas exchange. Orbital shaking incubators provide a temperature-controlled environment and agitation at variable speeds to incorporate oxygen and evenly distribute nutrients throughout the culture media.

Challenges with Shake Flasks

While shake flask cultures are easy to establish in a lab, the technical aspects of cultivating bacteria in them typically are underestimated as energy input and gas/liquid mass transfer are not well controlled. Flow conditions in shake flasks influence the cultivation of microorganisms as well as process variables such as mixing time, hydromechanical stress and maximum energy dissipation rate [5]. As such, processes for recombinant protein production often run under suboptimal conditions resulting from missing or incomplete information due to the lack of online monitoring and control systems. As a result, shake flasks have limitations in batch cultivation including insufficient aeration and fast nutrient depletion that results in lower cell density.

Studies show that online monitoring tools for shake flasks are extremely helpful for determining optimal conditions and increasing yields [6]. However, measurement of conditions in shake flasks are mostly obtained offline by invasive sampling methods. They do not offer real-time or online measurement and control over process parameters that labs seek such as oxygen and carbon dioxide transfer rate, optical density, and pH.

Offline sampling also can have side effects on cultivation conditions that are, for example, affected by a drop in dissolved O_2 (DO; dissolved oxygen) or a shift in temperature [7]. Because of their limitations, shake flasks are more advantageous for screening or production of starter cultures. In applications where a well-controlled environment is required for providing high product yields with high reproducibility of individual cultures and scalability, different solutions are necessary.

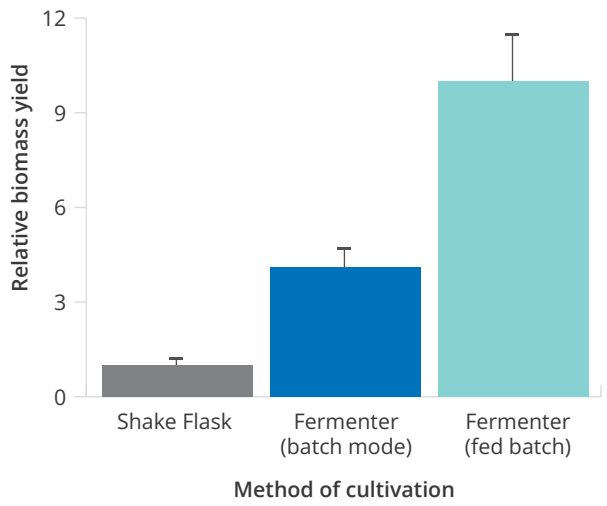
Bioreactors (Fermenters) Provide a More Controlled Environment for Protein Production

Fermenters address many of the limitations of shake flasks while offering greater control over biological processes for optimized results. Equipped with sensors, advanced software and control technologies and sophisticated gassing of the microbial culture, a modern fermenter establishes and sustains an ideal operating environment with conditions optimized for cellular growth and product formation, crucial for reproducibility and high-quality yields.

Advanced sensors integrated into a fermenter precisely regulate and constantly monitor crucial parameters such as pH, temperature, oxygen and nutrient consumption in an automated way. The sensors provide a wealth of information on growth parameters in real time. Process kinetics by mathematical models can help improve scalability, yield, and efficiency of the fermentation process. Optimizing all parameters creates an environment that is ideal for the specific needs of the biological entity inside the fermenter.

For example, yields in an overnight culture of a bacterial biomass (see Figure 1) can be up to four times higher using a bioreactor as a fermenter compared to a shake flask. Using fed-batch feeding regimes, this factor can be further improved to as high as 10 times. The initial investment in a bioreactor (fermenter) is well compensated for improved productivity, reduced hands-on time and bench-space and much higher reliability and reproducibility of the protein production process [8].

Figure 1



Comparison of yields using a shake flash vs. fermenters.

High yield production of recombinant proteins in large scale fermenters is well established in the biotechnology industry. Pharmaceutical, food, and chemical companies have been quick to utilize the technology because of better scalability, process control, and a more economical approach to protein production. Currently, more than half of the fermenter applications are in industry as well as in academic institutions, with the majority using lab-scale or pilot-scale vessel sizes, indicating the increasing usage of small-scale fermenters [9].

In addition, fermenters address obstacles in scaling biological processes associated with shaker incubators, enabling the transfer from small-scale experimental vessels to large-scale production units without losing the defining characteristics of the process. This level of precision during scale-up is important for the consistent quality of the end product in processes where larger amounts of protein are required. Basic fermentation vessels, like shake flasks, cannot consistently replicate conditions at larger scales and require multiple vessels that take up space.

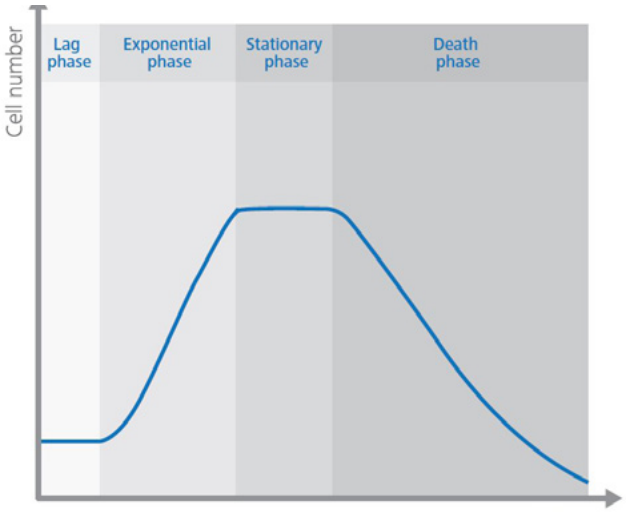
Based on these capabilities, a fermenter is not just a vessel for growth, but a dynamic tool that propels the biotechnology industry forward. It marries the biological and mechanical realms to foster the continual advancement of cellular and microbial production technologies.

Fermentation Process Modes Not Available in Shake Flasks

A major advantage of fermenters over a shake flask culture is the option to use it in several operation modes. Bacterial cell growth in a fermenter is dynamic (See Figure 2), involving several phases.

At inoculation, cells adjust to the new environment with a slow growth. Following the adaptation period (lag-phase), cell division reaches its maximum and nutrients and O_2 are metabolized at the maximum rate (exponential phase). Increasingly produced amounts of waste products affect the pH and slow bacterial growth (stationary phase). At this stage, it is important to maintain conditions that best support cellular growth. Unreplenished nutrients eventually increase the rate of cell death (death phase). As bacteria decompose, free cellular proteins lead to foam formation.

Figure 2



Growth phases in a bioreactor (fermenter)

Unlike shake flasks, fermenters offer different process operations with varied substrate feeding protocols to maintain optimal process conditions for specific cell cultures. The choice of bioprocess operation depends on the cell growth strategy.

Three basic modes of operation include:

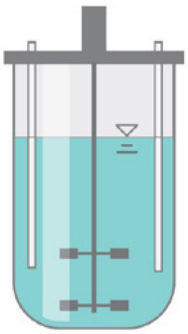
- Batch
- Fed-batch
- Continuous culture

The choice of bioprocess operation depends on the cell growth strategy.

Batch

The simplest mode is batch operation (Figure 3). This closed process contains all the nutrients at the start of fermentation without additions of substrate or medium (except for gasses for aeration and pH management). No extra feeding takes place. The process resembles a shake flask approach but provides significantly higher yields.

Figure 3

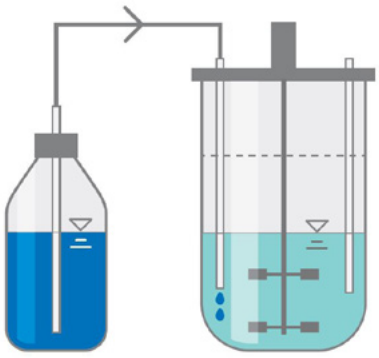


Batch process

Fed-Batch

In fed-batch mode (Figure 4), fresh medium and supplements are supplied during cultivation before nutrients become limited. This semi-continuous process maintains constant conditions and has less downtime as a longer process. As the rate of feed determines growth rate, batch feeding allows for increased product quantities with greater cell densities as compared to batch processes. This process mode is a key advantage of fermenter-based protein production over shake flasks in yielding significantly higher product yields. However, it is important to consider the inhibition caused by the accumulation of non-removable toxic by-products.

Figure 4



Fed-batch process

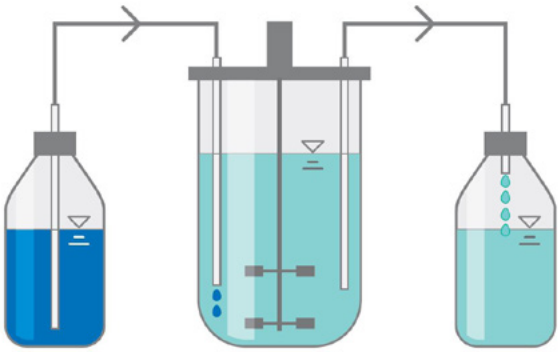
Continuous Culture

In continuous culture (Figure 5), an equilibrium or steady state is created by continuously adding fresh culture medium, while keeping the cell concentration constant. As medium is removed and cells are discharged both inflow and outflow rates must be less than the doubling time of cells. This strategy is particularly suitable when an excess of nutrients would result in the inhibition of cell growth.

As with fed-batch mode, the continuous process mode cannot be implemented in shake flasks. In continuous culture, a smaller fermenter vessel can yield the same or higher amounts of product than a larger vessel used in a batch process. Hence, vessel volume no longer is the major aspect for scalability. In contrast to shake flask culture, scaling the process has no or very little impact on bench space or hands-on time.

This production mode is very attractive and widely used in the biotechnological industry, especially when mammalian cells are used as the host cells in fermenters. Another benefit is the ability to use smaller reactor vessels to produce amounts of protein equivalent to those produced in (fed) batch processes.

Figure 5



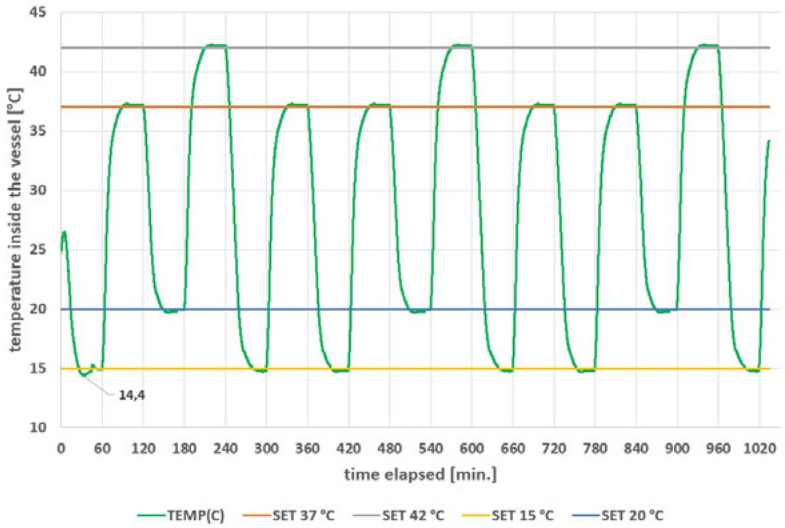
Continuous Process

Bioreactors (Fermenters) Monitor Bioprocess Parameters

Sensors provide data that enables real-time monitoring and ongoing adjustments of perfect growth and protein production conditions in an automated way. Shake flash cultures are completed blindly, without the means to continually optimize conditions. Below are examples of different sensors implemented in modern fermenters to measure, control and record important bioprocess parameters.

Figure 6

Alternating temperature profile of 15°C, 20°C, 37°C, 42°C in a 1L double wall vessel (IKA Habitat with HRC2 lite thermostat) shows heating and cooling times of the bioreactor (fermenter) medium (LB broth).



Temperature

As cells and microorganisms operate optimally at specific temperatures, staying within a limited temperature range is important for ideal protein production. While heating is typically required during the early exponential phase of bacterial cultures, cooling the fermenter is typically required during the intense growth of later stages and in larger vessel volumes (See Figure 6). The temperature in fermenters can be measured by various sensor types such as resistance temperature detectors (RTDs), or infrared thermometers. Reaching the desired temperature quickly and maintaining close control is important.

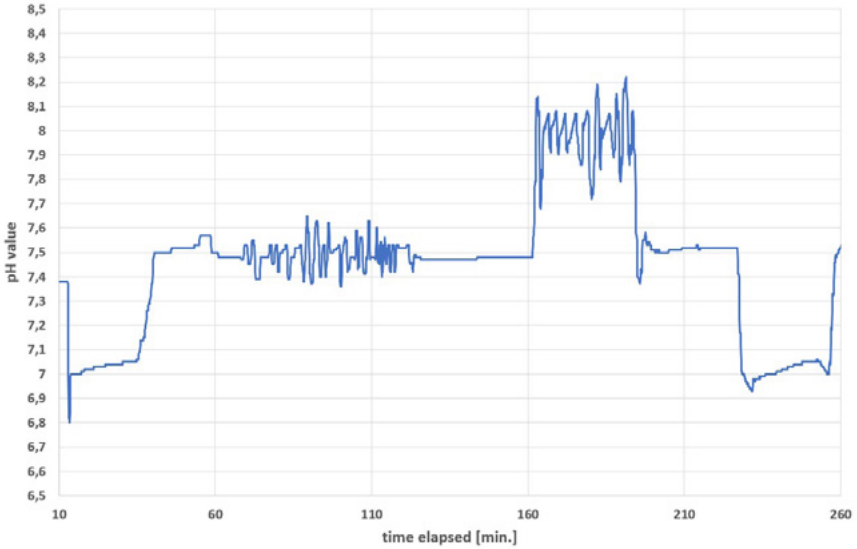
pH

While pH typically cannot be regulated in a shake flask culture, maintaining an optimal pH level in the fermenter is crucial to avoid detrimental effects on cell physiology. Keeping an optimal pH level in the fermenter is a delicate endeavor as the slightest deviation can amplify effects on cell physiology (see Figure 7). pH influences enzyme activity, nutrient uptake, and cell structural integrity. Its precise manipulation in response to the metabolic excretions of the culture ensures an environment conducive to ideal cell growth and productivity. Degradation products of nutrients consumed by the protein expressing cells can change the pH value inside the fermentation broth.

To adjust the system back to the ideal pH value, repetitive small doses of acid or base solution are added according to the reading of the pH sensor. Electrochemical glass pH sensors are traditionally used in bioprocessing for continuous pH measurement, but also optical sensors are available.

Figure 7

pH dynamics in a bioreactor (fermenter) (0.5L vessel with PBS buffer, IKA Habitat, standard tubing with 3.2 mm ID) achieved with 1M HCl & 1 M NaOH for pH adjustment. The pH value was effectively regulated to different setpoints close to the edge of the pH buffer zone (pH 8, set value +/- 0.05 pH). Regulation at pH 8 is challenging because pH8 is out of the buffers' range, pH 7 and 7,5 can be held precisely with the use of very little acid and base.



Dissolved Oxygen

Dissolved oxygen (DO) is a significant parameter affecting culture growth in fermenters. As cells rely on oxygen for their respiratory needs, its scarcity affects cell growth and product synthesis.

In a shake flask culture, the aeration can be impacted only by manipulation of the orbital shaker speed or using baffled vessels. A measurement of DO typically is not possible. In fermenters, the interplay between oxygen supply and cellular uptake is monitored by advanced DO sensors, which feed back into the control system to ensure oxygen is neither a limiting factor nor supplied to deleterious excess.

In principle, there are two types of DO sensors:

1. Polarographic DO sensors measure the electrical current within the solution to infer the oxygen concentration.
2. Optical DO sensors measure the luminescence of a solution using blue light, with the presence of dissolved oxygen decreasing the luminescence.

Exhaust Gas

Exhaust gas composition originating from the cells' metabolism provides a wealth of additional information. On-line analysis of exhaust gas (off-gas) composition, especially O_2 and CO_2, enables the calculation of key bioprocess variables such as oxygen uptake rate (OUR), carbon dioxide evolution rate (CER), and respiratory quotient (RQ) and enable the scientist to understand the metabolism of the cells during the fermentation process.

Analysis of off-gas composition is possible with fermenters but not with shake flask. It provides information that is essential for process optimization, control, scaling, and validation. Off-gas analyzers use a combination of infrared light sensors or galvanic sensors to determine CO_2 or O_2 content.

Foam

When rates of cell division are high, nutrients and O_2 are metabolized quickly with cells, producing proportionally increasing amounts of waste products. When the cells are growing fast, a significant amount of oxygen needs to be transferred into the fermenter and the mixing speed is often high. This generates foam, which may clog the filter and ultimately lead to an overfilling of the fermenter. In a shake flask culture, the shaker speed must be reduced to avoid foaming out of the flask, which leads to suboptimal oxygen levels in the medium.

In contrast, maintaining the right liquid level in a fermenter without exceeding a reasonable amount of foam can be achieved without compromising DO levels. This is accomplished through the automatic addition of surface-active agents called "anti-foam". Optical, contact-free foam detectors attached to the outside of the fermenter vessel are ideal for this purpose and may be combined with an electrical level sensor to maintain constant liquid level while controlling foam formation by regulating agitation speed.

Filling Level

When feeding or removing medium to/from the fermenter, it is critical to closely control the liquid volumes, which can be achieved through calibrated pumps regulated by the fermenter's controller or by employing gravimetric feeding methods. Gravimetric feeding is a very accurate method to control the amount of medium added to a fermenter. It uses a balance instead of a volume measurement for precisely controlling the flow of feeding liquids into a fermenter

For measuring the fill level of a fermenter, a conductive level probe is the most robust and economical option for level control. The sensor is mounted on the vessel lid and reacts to contact and non-contact with liquid in the reactor.

Biomass and Cell Viability

Biomass monitoring is considered an essential parameter for controlling fermenter conditions. In a shake flask culture, the measurement of biomass relies on interrupting the process and drawing an offline sample creating suboptimal growth conditions. This increases the risk of contamination.

For fermenters equipped with a biomass sensor, the total cell concentration can easily be plotted over time by measuring the turbidity of the media solution. Plotting total biomass in a reactor over time yields an organism and process-specific growth curve to understand and optimize the induction time and following production of the desired product.

Sensors for biomass monitoring typically are optical sensors that determine the cell concentration either by measuring the amount of backscattered light from cells or quantifying the absorbance of light between a light source and a detector. Specific sensors are available to distinguish dead and live cells.

Optimizing the percentage of live cells in a fermenter process is important for optimizing growth and product yield. Cell viability sensors typically measure the integrity of cellular membranes to distinguish live cells with intact membranes from dead cells with disrupted membranes.

Figure 8

The figure represents a common setup of the HABITAT research laboratory reactor from IKA. As the first bioreactor with a lid stand, it ensures ergonomic working and a well-organized laboratory.

Making the Shift from Shake Flask to Bioreactor (Fermenter) Technology

Moving from a shake flask culture to a fermenter system can create concerns due to the different bioprocessing principles. However, current technology allows for an easy transfer from a shake flask to a fermenter-based culture system.

The following steps show the simplicity of setting up and running a bacterial culture within a fermenter in batch mode. Figure 9 shows an example of a finished fermenter bench set up for a planned DNA transformation experiment.

1. To prepare the fermenter, the vessel is assembled with the stirrer, sparger and sensors. It is then filled with medium. The system guides the operation though the calibration of sensors and pumps which should be executed regularly with a few easy steps.
2. Once the vessel is assembled and filled, the entire configuration, including acid & base and gas lines, is transferred to an autoclave and sterilized with a typical protocol (121°C, 1 bar, 20 min). During sterilization, the gas supply is configured by connecting O_2, compressed air or N_2 lines to the controller. If compressed air is not available in the lab, a mobile compressor system provides a cost-effective alternative. Typically, gases are supplied at two bars to the fermenter controller.
3. Once sterilization is completed and the medium has cooled, acid, base and gas lines can be connected to the pumps or gas outlets of the fermenter controller. The software will guide the user through the process.
4. When initializing the run, the system will automatically adjust the pH and DO values and temperature to the desired setpoints. Once the specific conditions are reached, a sufficient number of bacteria (“starter culture”) that is still in the exponential growth state is added. It will meet perfect growth conditions from the start. This will reduce the lag phase.
5. After inoculation of the media in the fermenter vessel with a starter culture and initiation of the cultivation, the process is automatically controlled by the software based on the data delivered from the reactor’s sensors. Process data is automatically and constantly measured and stored for later review or reporting.
6. Once the determined point on the growth curve is reached, the cells can be induced for protein production.
7. At the optimal timepoint, the vessel content can be harvested from the fermenter through a harvest pipe.

Conclusion

When producing biomass or recombinant proteins, fermenters offer several compelling reasons to move from a shake flask culture. The major drawbacks of shake flasks are the limited control overgrowth conditions that influence the quality of microorganism cultivation and its limitation in volumes. Specifically, process variables such as DO, pH, carbon dioxide transfer rate, off-gas and biomass are not obtained online and in real time [7].

Using a shake flask, a failed culture usually is not recognized until after the prescribed incubation period. At the same time, varying conditions between flasks or between runs impact the reproducibility of yielded product. Most importantly, hands-on time can be substantial for shake flask culture, especially if the process is scaled and many flasks are to be handled. This entails cleaning, autoclaving, inoculation and harvesting for each single flask.

Fermenters provide multiple advanced capabilities compared to a shake flask to automatically control the bioprocess, avoiding the occurrence of unfavorable conditions to occur during culture. As a result, stirred tank fermenters provide high reproducibility of biomass production and product yield. Additionally, increased control of a single parameter or a complete process control allow for control strategies not possible with shake flasks and ultimately lead to reliable, reproducible and easy to evaluate bioprocesses that easily scale into sometimes very large volumes (e.g. 10000 L).

IKA Simplifies the Transfer to a Bioreactor (Fermenter)

The process of moving from shake flasks to a fermenter must be as simple as possible since lab personnel have different levels of expertise. With an exceptional user-friendly interface, intuitive software, sensor interfaces and tablet compatibility, the IKA HABITAT fermenter increases process control while making work easier. Equipment handling and process operations are simple, shortening the learning curve. Students and novice researchers can quickly learn to use the fermenter for research and studies. Experiments can easily be repeated by different individuals. Virtual reality training enables learning from anywhere, anytime at a person's own pace.

IKA combines the latest technology with innovative design and thinks far into the future. As both a responsible family business and a global player, over 900 employees contribute to expanding the leading global market position in laboratory, analytical, and process technology every day. At the headquarters in Staufen, Germany, products and technologies are developed in cooperation with application experts from science and industry.

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