Controlling Nutrient Release in Cell Cultivation

EnBase Designed to Optimize Feeding in the Production System

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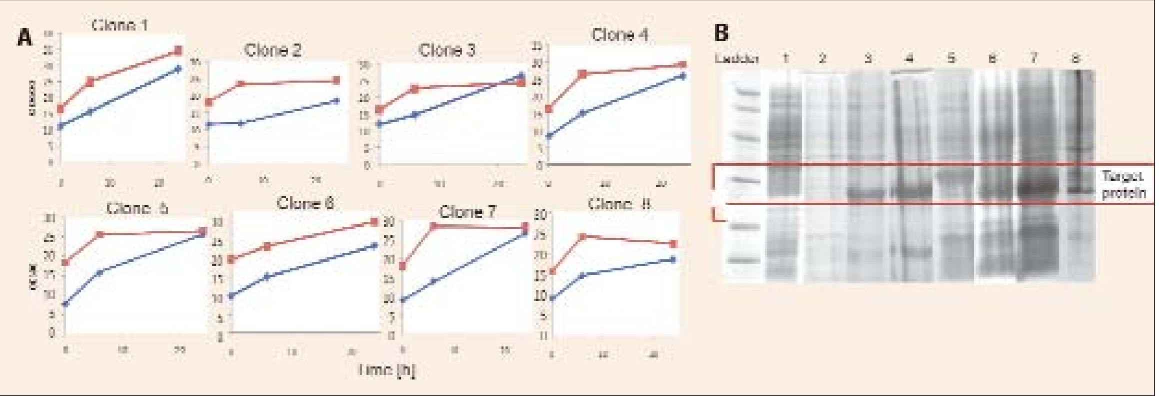

Figure 3. Growth curves: (A) EnBase cultivations with *E. coli* clones derived from the Human Protein Atlas project and their target proteins (B) analyzed by SDS-PAGE

With the arrival of the post-genomic era, functional genomics and proteomics have been fields of intense research. Extensive investigations on thousands of novel proteins to understand their function and interactions in biological systems have increased the need for the development of high-throughput methods. The need for reliable and efficient technologies suitable for parallelization or automation is urgent.

BioSilta's (www.biosilta.com) EnBase™ has applicability for high-throughput protein production as well as scale-up of industrial bioprocesses.

Small-scale cultivation in shake flasks and microwell plates is usually performed as a batch culture, which may lead to oxygen limitation, medium acidification, and overflow metabolism. As a result, only low cell densities with OD_{600} from 1 to 10 are obtained.

High cell density cultivation of microorganisms in a bioreactor is an efficient method for achieving a high specific productivity where final cell densities up to an OD_{600} of 400 can be obtained in the cultivation of *E. coli*.

One problem that has to be addressed in high cell density cultivation is the increasing demand for oxygen, this is problematic as oxygen's solubility decreases with growing cell densities. To avoid the problems that can occur with the use of pure oxygen, cultivation can be carried out with lower specific growth rates.

The most exploited technique used to achieve high cell densities in *E. coli* cultivations is fed-batch fermentation. Once the growth-limiting substrate has been consumed, a feeding solution containing the concentrated substrate is continuously added. During the fed-batch process, it is critical to control the specific growth rate as the formation of inhibitory by-products, cell productivity, and plasmid stability are all related.

Adept control of reaction rates avoids problems associated with cooling and oxygen transfer. Furthermore, careful oversight of metabolic processes avoids osmotic effects, catabolite repression, and overflow metabolism. Scale-down is a challenging task when one views reactor configuration in relation to sensor technique, high viscosity of the feeding solution, continuous substrate delivery, mixing on small scale, and precise feed control in parallel cultures.

EnBase technology enables the feeding of glucose in closed systems for small-scale cultivation under fed-batch conditions. The slow release of glucose into the cultivation medium supplies the bacterial culture with limiting substrate. A biocatalytic reaction controls the supply of glucose, which is derived from a polymeric substrate embedded in a gel and diffused into the medium or dissolved directly in the medium.

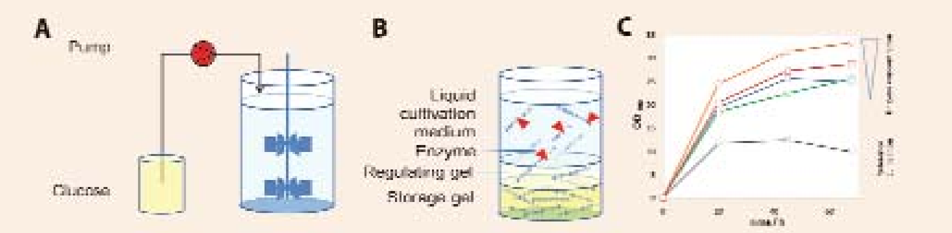

Figure 1. Fed-batch cultivation in a bioreactor (A) and an EnBase cultivation vessel (B): Cell growth of *E. coli* (C) under EnBase fed-batch conditions using different enzyme concentrations compared with cell growth under batch conditions (reference cultivation)

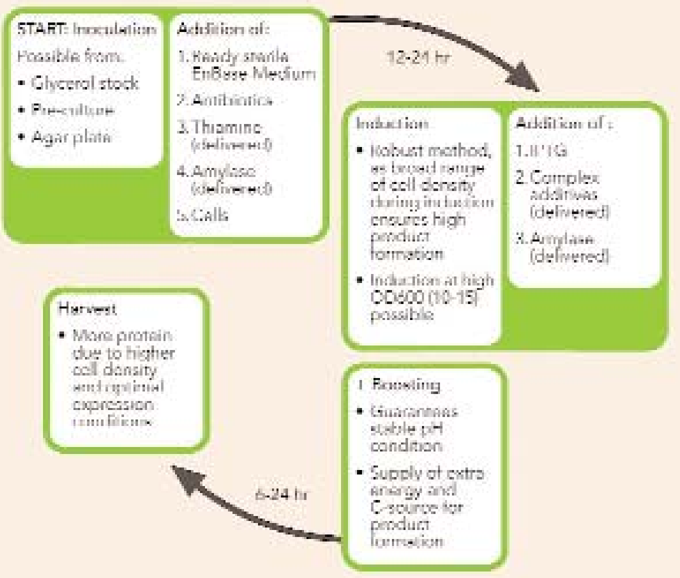

Figure 2. Two-phase cultivation with EnBase: In the first step, cells grow in fed-batch mode controlled by the addition of amylase. In the second phase, medium optimized for protein expression is supplied after addition of boosters. Balanced addition of C- and N-source during induction increases the final volumetric product yield.

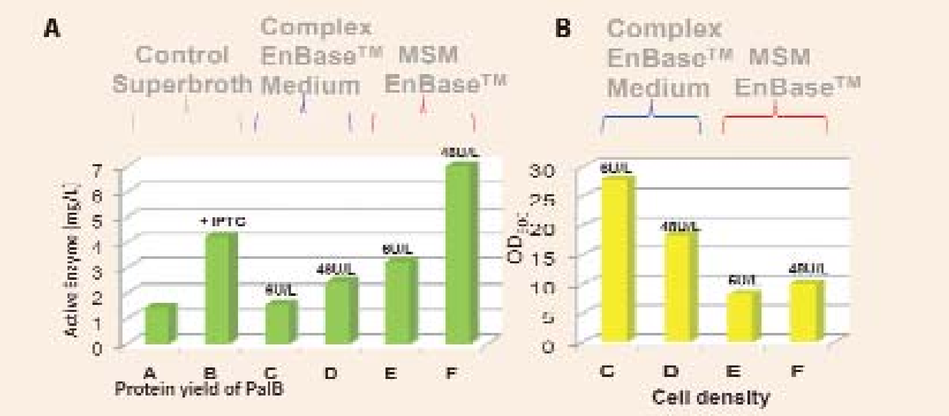

Figure 4. Comparison of protein yield (A) of PalB obtained with standard medium and EnBase medium: Cell densities (B) obtained in EnBase 24 deep-well plates

The enzyme functions like a pump in the bioreactor (*Figure 1A*) to control glucose supply and turns a shake flask, microwell plate, or deep-well plate into an efficient bioreactor system (*Figure 1B*). The cell growth rate can be controlled by using different enzyme concentrations (*Figure 1C*).

Recombinant protein expression with EnBase is performed in a two-phase cultivation (*Figure 2*). In the first step, cells grow in a fed-batch mode controlled by the addition of an amylase. Balanced growth and favorable pH after overnight cultivation make the bacteria ready for efficient recombinant protein production.

In the second phase, medium optimized for protein expression is supplied after the addition of boosters. It is possible to induce recombinant protein production at high cell densities without sacrificing the protein productivity per cell because medium conditions and the physiological state of the cells can be kept optimal.

EnBase works well with induction cell densities of OD_{600} of 5 to 15 and expression times of up to 24 hours. The balanced addition of carbon and nitrogen during induction increases the final volumetric product yield.

Recombinant Protein Production

Higher volumetric product yields enable scaling down of the protein production process, saving time, space, and manpower. As part of the Human Protein Atlas project, nearly 300 different recombinant proteins (with a range of 25–150 amino acids in length) are produced per week. The protein fragments are fused with an N-terminal hexa-histidine albumin binding protein tag and expressed in *E. coli* Rosetta (DE3) cells. The cells are cultivated in 100 mL

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complex medium in 1 L shake flasks.

To reduce the manual handling steps and increase the throughput, 15 randomly selected clones were tested in EnBase 24 DWP (deep-well plates) using two different EnBase media. With the EnBase DWPs the final cell densities reached an OD_{600} of 25 (*Figure 3A*) as compared to an OD_{600} of 6, which was the average cell density in the shake flasks.

The protein yields from preliminary data (*Figure 3B*) obtained with the blue medium looks promising and indicate that one 24 DWP could substitute for six shake flasks.

Slow controlled cell growth in balanced medium supports the correct folding of translated proteins as shown in *Figure 4B*.

Recombinant expression of *Pseudozyma antarctica* lipase B was investigated by comparison of different expression systems in *Pichia pastoris* and *E. coli* strains. Expression (*Figure 4*) was performed in the periplasmic space of *E. coli* Rosetta (DE3) using a 1 L shake flask with superbroth medium, which resulted in 4 mg/L of active enzyme.

The highest enzyme activity value was obtained when EnBase mineral salt medium (MSM) was used for cultivation. High cell density did not guarantee high protein yield, however. Instead the EnBase MSM with 48 U/L amylase showed a yield of 7.0 mg/L (*Figure 4A*), but had a low cell density of OD_{600} of 10 (*Figure 4B*). The higher yield of active enzyme was reached due to a higher amount of soluble protein per cell.

Efficient small-scale production of recombinant proteins without the use of fermentors affords wide application in structure-based drug design, antibody production, and screening of protein libraries. Screening of different parameters such as strains, vectors with specific promoters, ribosome binding sites, fusion tags, purification tags, media, and additives or induction mode can also be easily performed in parallel in deep-well plates.

Investigations enabled by Enbase include many process development steps, including determination of glucose gradient by setting glucose pulses, influence of oxygen parameters, or effect of induction time under fed-batch conditions. Although the technology was developed for *E. coli*, but preliminary studies have shown its feasibility for *Bacillus subtilis, Lactobacillus brevis,* and *Pichia pastoris*.

As the EnBase technology is an appropriate tool for high cell density cultivation in 96 well plates, its application in high-through put screening of metagenomic or mutant libraries is logical. As such, BioSilta has developed a specific EnBase medium for the auxotrophic *E. coli* mutants DH5 alpha and DH10 beta.

High-throughput sequencing, high-throughput crystallization approaches, and production of labeled proteins for NMR studies are additional applications.

One interesting future project is the application of EnBase technology to Wave single-use bioreactors. Cell bags are placed on a special rocking platform to provide mixing and oxygen transfer. As oxygen transfer is limited in Wave bioreactors the EnBase system could control the cell growth and adapt it to the existing oxygen conditions. GEN