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# Investigating expression systems for the stable large-scale production of recombinant L-leucine-dehydrogenase from *Bacillus cereus* in *Escherichia coli*

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**Abstract** The established *Escherichia coli* expression vectors ptrc99a, pKK223-3, pPL$\lambda$, pAsk75, pRA95, and pRA96, which differ in copy number, mode of induction, selection marker, and use of *par* sequences for stabilization, were investigated for the stable expression of recombinant L-leucine dehydrogenase from *Bacillus cereus* with a view to large-scale production. Best results were achieved with pIET98, a runaway-replication system derived from pRA96. Expression of L-leucine dehydrogenase was controlled by its constitutive *B. cereus* promoter and depended on host strain, cultivation temperature, induction time, and media composition. After cell cultivation at 30 °C and shifting to 41 °C to induce plasmid replication, *E. coli* BL21[pIET98] yielded 200 U LeuDH/mg protein, which corresponds to >50% of the soluble cell protein. Continuous cultivation in a semisynthetic high-cell-density medium verified structural and segregational stability over 100 generations in the absence of a selection pressure.

## Introduction

The development of useful recombinant microorganisms for production processes requires close interaction between the genetic engineer and fermentation technologists or biochemical engineers throughout the cloning period, to ensure that the characteristics of the host-vector combination are suitable for a large-scale operation. The primary aim is to achieve a production strain with high specific productivity and stability. In addition, there is increasing competition with regard to production costs. These parameters are influenced by the choice of host organisms (Ohta et al. 1993) and expression systems (Balbas and Bolivar 1990) as well as by the growth conditions (Fieschko 1989) and the cultivation process (Knorre et al. 1990). Unfortunately, these influences are numerous, they often interact in an unpredictable manner, and results are seldom reported in the form of a systematic comparison. To date it remains necessary to empirically optimize single expression systems for individual proteins.

In this study we investigated the possibility of overexpressing L-leucine dehydrogenase (LeuDH) from *Bacillus cereus* in *Escherichia coli* with respect to technical application using established expression vectors and host strains readily available. The great variety of expression vectors inducible by isopropyl-$\beta$-thiogalactoside (IPTG) were represented by the *E. coli* expression vectors pKK223-3 and pTrc99a. In addition, the expression of the recombinant gene was induced by shifting the cultivation temperature to 41 °C using pPL$\lambda$ and by the addition of anhydrotetracycline to the culture medium using pASK75, which is able to secrete the recombinant protein into the periplasmic space. pRA95 and pRA96 represented vectors which start replicating when the cultivation temperature is shifted (Nordström and Uhlin 1992).

LeuDH (E.C.1.4.1.9) from *B. cereus* is an NAD(H)-dependent oxidoreductase, catalyzing the reductive amination of a variety of aliphatic 2-oxo-acids to the corresponding L-amino acids. It is of special interest as a catalyst for the industrial production of nonproteinogenic L-amino acids with bulky side chains such as L-tertiary leucine or L-neopentylglycine (Bommarius et al. 1995; Krix et al. 1997) and is therefore demanded in large amounts. *B. cereus* is not a useful production strain as it may produce toxins. The structural gene for *B. cereus* LeuDH had previously been cloned into *E. coli* by Stoyan et al. (1997) and was utilized in this study.

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## Materials and methods

### Bacterial strains and plasmids

The organisms used in this study were the *E. coli* strains BL21 [*hsdS gal* (*λIts857 ind1 Sam7 nin5 lacUV5*)], DH5([*supE44* (Δ*lac U169*

*ϕ80lacZΔM15*) *hsdR17 recA1 gyrA96 thi-1 relA1*], HB101 [*supE44 hsdS20*($r_B^- m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 cyl-5 mtl-1*], JM105 [*supE endA sbcB15 hsdR4 rpsL*($Str^r$) *thi* Δ(*lac-proAB*) *F′*(*traD36 proAB*$^+$ *lacI*$^q$ *lacZΔM15*)], JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) *F′*(*traD36 proAB*$^+$ *lacI*$^q$ *lacZΔM15*)], N4830–1 [$F^-$ *galK8 thi1 thr1 leu36 lacY1 fhuA21 supE44 rfbD1 morA1 his ilv* Δ(*hemF-esp*) Δ(*bio-uvrB*) *λ*[Δ*Bam* Δ(*cro-attR*)] $N^+$ *cI857*], SG13009 [($Nal^S$ $Str^S$ $Rif^S$) *lac*$^-$ *ara*$^-$ *gal*$^-$ *mtl*$^-$ $F^-$ *recA*$^+$ *uvr*$^-$ (*pRep4, Neo*$^R$)], XL1-Blue [*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$ $F^-$(*proAB*$^+$ *lacI*$^q$ *lacZΔM15 Tn10tet*$^R$)] and were obtained from Boehringer Mannheim (Mannheim, Germany), Pharmacia Biotech (Uppsala, Sweden) and Stratagene (Amsterdam, The Netherlands).

The *E. coli* vectors pKK223-3, pTrc99a, pPL*λ*, and pUC18 were obtained from Pharmacia Biotech. pASK75 came from Biometra (Göttingen, Germany). pRA95 and pRA96 were supplied by Nycomed Pharma (Copenhagen, Denmark).

Enzymes for DNA cloning were purchased from Pharmacia Biotech, Boehringer Mannheim (Mannheim, Germany), and New England Biolabs (Schwalbach, Germany). Extraction of plasmid DNAs from *E. coli* strains, purification of PCR products, and extraction of DNA fragments from agarose gels were performed using appropriate commercial kits (Biorad, Krefeld-Oppum, Germany; Quiagen, Hilden, Germany). Molecular cloning was performed according to standard protocols (Sambrook et al. 1989).

### Media

Cells were grown in Luria-Bertani (LB) medium (Sambrook et al. 1989) and high-cell-density (HCD) medium, respectively, containing appropriate antibiotics at concentrations of 100 mg/l ampicillin or 12.5 mg/l tetracycline. HCD medium consisted of glucose (6 g/l) $MgSO_4 \cdot 7H_2O$ (1 g/l), yeast extract (3 g/l), $NH_4Cl$ (0.2 g/l), $(NH_4)_2SO_4$ (2 g/l), $KH_2PO_4$ (13 g/l), $NaH_2PO_4$ (5.3 g/l) NaEDTA (8.4 mg/l), thiamine (0.1 mg/l), vitamin solution 428 (DSMZ, Braunschweig, Germany) (5 ml/l), and trace elements (4 ml/l). Thiamine was sterilized separately by filtration. The trace element solution contained $CaCl_2 \cdot 2H_2O$ (40 mg/l), $ZnSO_4 \cdot 7H_2O$ (2 mg/l), $CuCl_2 \cdot 2H_2O$ (1 mg/l), $MnSO_4 \cdot H_2O$ (10 mg/l), $CoCl_2 \cdot 6H_2O$ (7 mg/l), $H_3BO_3$ (0.5 mg/l), $AlCl_3 \cdot 6H_2O$ (10 mg/l), $Na_2MoO_4 \cdot 2H_2O$ (2 mg/l), and $FeSO_4 \cdot 7H_2O$ (40 mg/l) solubilized in 5 *N* HCl.

### Cultivation and growth determination

Growth of *E. coli* cells was initiated by streaking −80 °C stock cultures on an LB agar plate containing appropriate amounts of antibiotics. The plate was incubated at 30–37 °C until single colonies appeared. Shake flasks containing LB or HCD medium, respectively, were inoculated by picking single colonies or by adding 1% (v/v) of an appropriate preculture. Cells were incubated at 30–37 °C and 110–190 rpm on a rotary shaker to the desired densities. Cell densities were determined by measuring the absorption at 660 nm using a UV/VIS spectrophotometer (Shimadzu, Kyoto, Japan). Continuous cultivation of recombinant organisms in shake flasks was performed by inoculating LB or HCD medium lacking antibiotics with 1% of a preceding culture, growing this to stationary phase, and inoculating the next culture.

### Enzyme assay and protein determination

Enzyme activity was measured at 340 nm and 30 °C using a UV/VIS spectrophotometer (Shimadzu, Kyoto, Japan). The standard assay mixture for the reductive amination reaction contained 4.5 mM sodium 2-ketoisocaproate and 0.204 mM NADH in 900 mM $NH_4Cl$ buffer (pH 9.5). The reaction was performed in a final volume of 1 ml and initiated by the addition of limiting amounts of enzyme. One unit of LeuDH was defined as the amount of enzyme that catalyzes the consumption of 1 μmol NADH per minute under standard assay conditions. Specific activity is expressed as units per milligram of soluble protein. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin for calibration.

## Results

### Plasmid construction

All genetic work was based on pLeu2, an *E. coli* vector constructed by Stoyan et al. (1997) inserting the *LeuDH* gene of *B. cereus* including its natural constitutive promoter into the established cloning vector pUC18. We found that the expression of pLeu2 in *E. coli* XL1-Blue was highly unstable in larger cultivation volumes (data not shown).

From pLeu2 the *LeuDH* gene was amplified and equipped with appropriate terminal restriction sites by PCR with the intention of minimizing the distance between the promoter of the various expression plasmids and the ATG codon of the recombinant gene. In addition, the *B. cereus* promoter sequences were removed for expression using pKK223-3, pTrc99a, pASK75, and pPL*λ*. The resulting PCR fragments were ligated into pUC18, sequenced and subsequently cloned into the appropriate expression vectors. The insertion of the *LeuDH* gene into the *Sma*I-*Pst*I sites of pKK223-3 resulted in the expression system pLeuA. The ligation into the *Nco*I-*Pst*I sites of pTrc99a yielded pLeuB. pLeuC was constructed by inserting the *LeuDH* gene into the *Hpa*I site of pPL*λ*. The in-frame orientation with the $P_L$ promoter was proven by restriction analysis (data not shown). pLeuD was generated by ligating the *LeuDH* gene to the *Bsa*I-*Pst*I sites of pASK75. The construction of pIET98 from pRA96 is illustrated in Fig. 1. Upstream of the *LeuDH* gene 449 base pairs were included containing the natural *B. cereus* promoter. In addition a tetracycline resistance gene derived from pBR322 was inserted. The orientations of both fragments were determined by restriction analysis. Later experiments did not reveal an impact of the orientation on the expression activities. By a corresponding strategy pLeuF was constructed from pRA95. It differs from pIET98 by the basal copy number per cell.

### Expression activities

The efficiency of the generated expression systems for LeuDH production was determined by measuring the specific enzyme activity in corresponding growth phases in six to eight host strains each at various inducer concentrations or induction times, respectively. The best results in this series of experiments are summarized in Fig. 2 in comparison to the original system *E. coli* XL1-Blue[pLeu2]. While the expression from pLeuC, pLeuD, pLeuA, and pLeuF yielded low LeuDH concentrations and did not match pLeu2, a specific LeuDH activity about 200 U/mg protein, corresponding to more than 50% of the soluble cell protein, was obtained by the

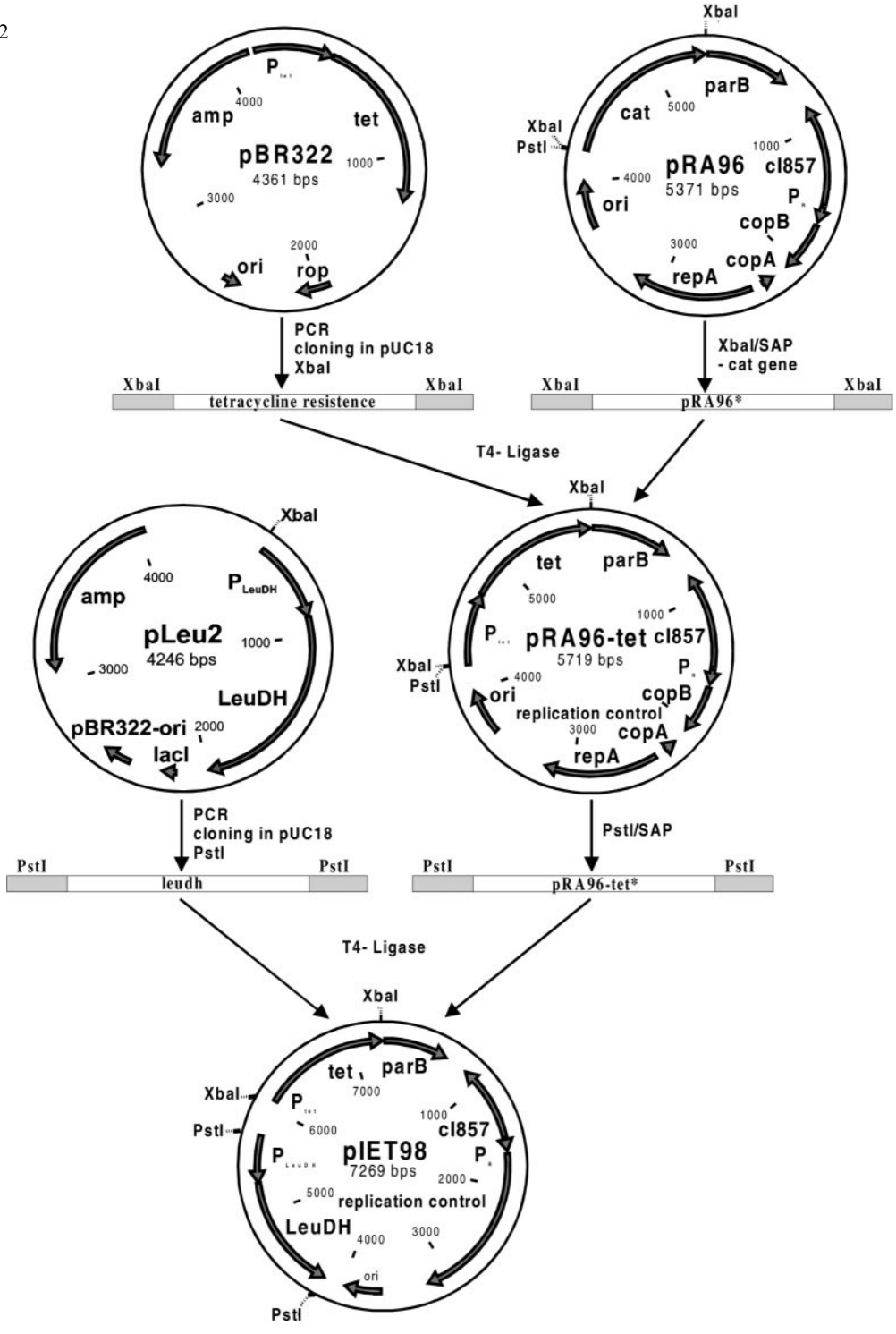



**Fig. 1** Construction of pIET 98 from pRA96, pLeu2, and pBR322

IPTG-inducible system *E. coli* JM105[pLeuB] as well as by the runaway replication system *E. coli* BL21[pIET98], exceeding the original expression system *E. coli* XL1-Blue [pLeu2] by 43%.

The runaway replication vector is characterized by a copy number of approximately one per cell during cultivation at 30 °C, increasing to 1000 per cell on induction of plasmid replication by shifting the cultivation temperature to 41 °C (Kidwell et al. 1996). Stability is promoted by a *parB* sequence as well as by a tetracycline resistance gene. Unlike ampicillin, tetracycline is not degraded by resistant organisms, which ensures constant selection pressure throughout the whole cultivation (Sambrook et al. 1989). As stability of the expression system is of great importance to cultivation in industrial scale, and since induction by temperature shift is cheap

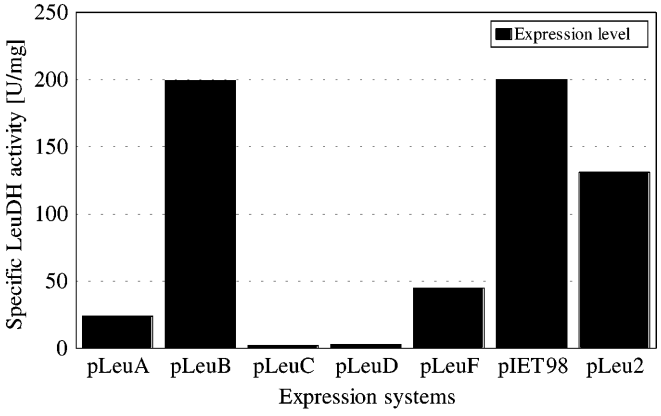


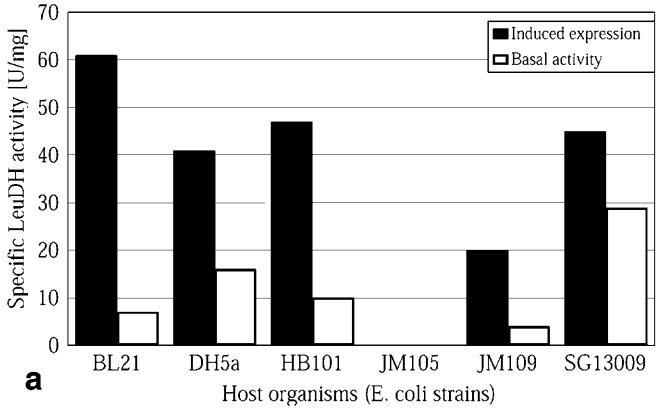

**Fig. 2** LeuDH expression activity using different optimized expression systems

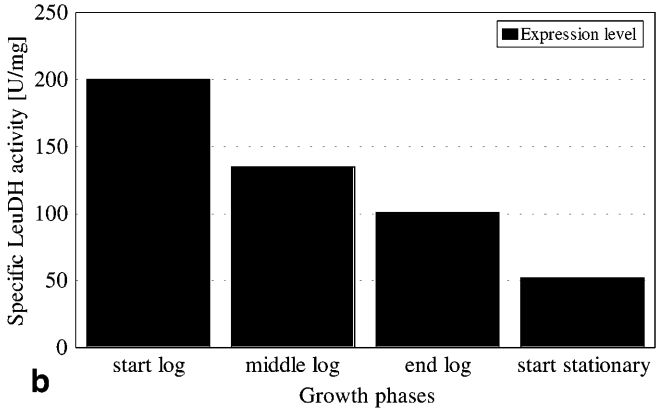
compared to chemical reagents like IPTG, pIET98 was chosen for further investigation.

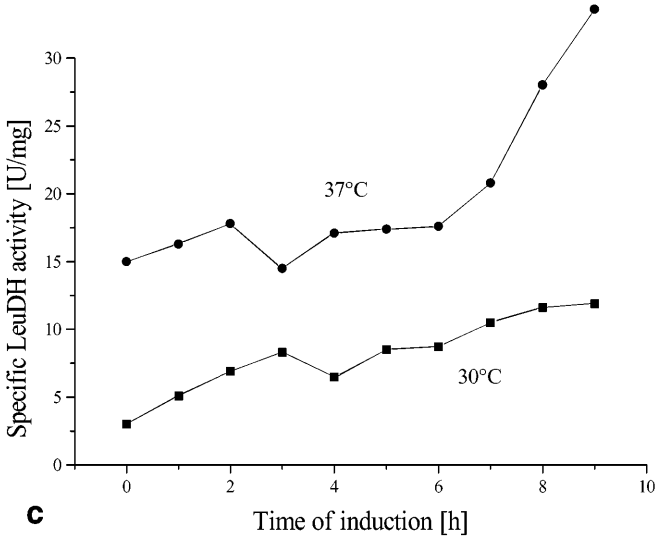
## Influences on expression activity

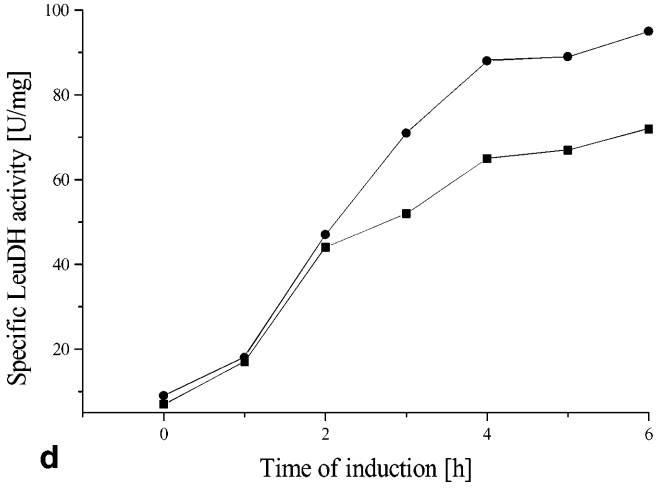
The expression activity of pIET98 was influenced by the choice of the host strain, the induction time, the cultivation temperature, and the media composition. As illustrated in Fig. 3, the host strain *E. coli* BL21 combining high expression activity with low basal activity under non-inducing conditions, induction at the beginning of the exponential growth, a cultivation temperature of 30 °C and the use of a semi-synthetic high cell density medium proved optimal for LeuDH production.

**Fig. 3** **a** Influence of different *E. coli* host strains on the repression of runaway replication and the LeuDH expression level of pIET98. A large difference between the basal LeuDH level at a cultivation temperature of 30 °C and the expression level after induction indicates the ability of a host strain to efficiently repress runaway replication and express LeuDH under appropriate cultivation conditions. **b** Influence of induction time on the LeuDH expression of pIET98 using *E. coli* BL21 as a host strain. Runaway replication was induced by shifting the cultivation temperature to 41 °C at the starting point, the middle, and the end of the exponential growth phase as well as after the beginning of stationary growth. **c** Influence of cultivation temperature on the LeuDH expression of pIET98 using *E. coli* BL21 as a host strain. The expression level and thus the repression of runaway replication at temperatures 11 °C and 4 °C below the inducing temperature were investigated over a period of 9 h. **d** Influence of media composition on the LeuDH expression of pIET98 using *E. coli* BL21 as a host strain. The cultivation was performed at 30 °C; runaway replication was induced at the beginning of the exponential growth phase over a period of 6 h



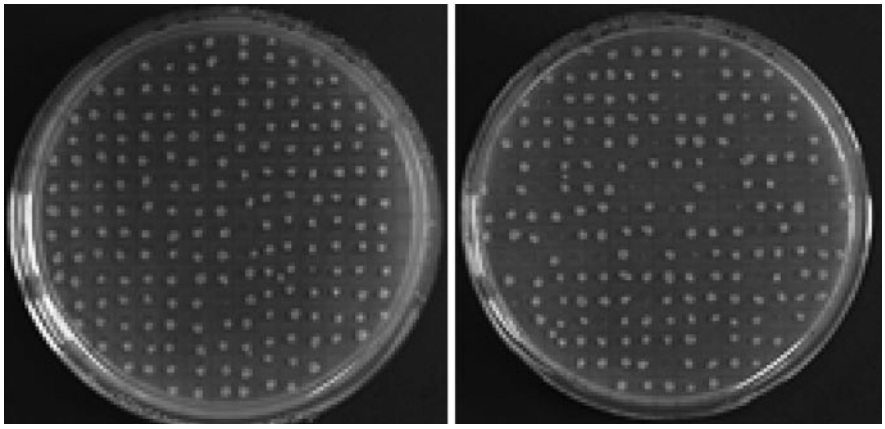


**Fig. 4** Plasmid loss after 100 generations cultivating *E. coli* BL21[pIET98] in LB (*right*) and HCDy (*left*) medium during continuous cultivation in the absence of antibiotics. Vacant places in the colony grid indicate *E. coli* BL21 cells that lost their plasmid during continuous cultivation and thus are no longer able to grow on media containing tetracyclin

Influences on stability

Figure 4 demonstrates the high segregational stability of pIET98 in *E. coli* BL21 over 100 generations in the absence of a selection pressure as well as differences in the use of complex LB and semisynthetic HCD medium for cultivation. Structural stability was determined by restriction analysis of pIET98 and proved to be unproblematic in either LB or HCD medium (data not shown).

## Discussion

Inserting the *LeuDH* gene of *B. cereus* into a variety of established *E. coli* expression vectors differing in copy number, mode of induction, selection marker and use of *par* sequences resulted in six expression systems able to yield active recombinant enzyme. The productivity of each system proved unpredictable; even closely related vectors such as pKK223-3 and pTrc99a, or pRA95 and pRA96, yielded very different amounts of recombinant LeuDH under corresponding growth conditions. Investigating pIET98, which combined high expression activity with an advantageous mode of induction, illustrated the importance of the choice of the host strain on enzyme productivity: while some *E. coli* strains were able to express large amounts of the recombinant enzyme at the same time as showing very low activity under noninducing conditions, others were either not able to express LeuDH or repress runaway replication. Additionally, the expression of recombinant LeuDH depended on the induction time. In contrast to established expression systems induced by adding IPTG in the middle of the exponential growth phase, runaway replication of pIET98 and thus LeuDH expression was best induced at the beginning of the exponential growth phase. Minimizing the expression of the recombinant enzyme under noninducing conditions required low cultivation temperatures, limiting plasmid replication at 30 °C. At the inducing temperature of 41 °C, control of plasmid replication is lost, increasing the gene copy number of LeuDH and consequently reaching high expression levels.

Besides high expression activity, stability of the recombinant expression system is of paramount importance for enzyme production on the large scale. It depends on the expression system as well as on the culture conditions. For pIET98, a much higher segregational stability was found in semisynthetic HCD medium than in complex LB medium, matching results found for other expressions systems (Mason and Bailey 1989). Because the expression activity of pIET98 was higher in high cell density medium than in LB medium, the use of HCD medium is indicated for LeuDH production.

In summary, *E. coli* BL21[pIET98] is a promising expression system for the large-scale production of LeuDH, combining high expression activity with high structural and segregational stability in the absence of selection pressure. Thus, it has the properties that are needed for a cheap and uncomplicated production process. Future investigations will deal with the suitability of the expression system for large-scale cultivation and adjustment of culture conditions if necessary.

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