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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Production of recombinant bacteriocin divercin V41 by high cell density *Escherichia coli* batch and fed-batch cultures

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Abstract To increase the yield of heterologous production of the class II bacteriocin DvnRV41 with *Escherichia coli* Origami (DE3) (pLysS/pCR03), induction of bacteriocin gene expression was optimized by varying the inducer isopropyl β-D-thiogalactopyranoside (IPTG) concentration (0–2 mM), and controlled batch and fed-batch cultures were tested on a 2-L scale. A concentration of 0.5 mM IPTG was found to be optimal for cell growth and bacteriocin production. Shake flask cultivation of *E. coli* Origami (DE3) (pLysS/pCR03) gave biomass and bacteriocin yields of 1.54±0.06 g cdw/l and 18±1 mg DvnRV41/l, respectively. Biomass (2.70±0.06 and 6.8±0.6 g cdw/l, respectively) and bacteriocin yields (30 and 74 mg DvnRV41 per liter, respectively) were both increased with batch and fed-batch compared to shake flask cultures. Bacteriocin yields reported in this study are among the highest published for other heterologous expression systems in shake flasks.

Keywords Divercin V41 · Bacteriocin · Heterologous expression system · Recombinant protein production

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Introduction

Bacteriocins are a subgroup of antimicrobial peptides with antimicrobial activity directed against related bacteria (Tagg et al. 1976). Although these antimicrobial compounds are naturally produced by many bacteria, bacteriocins from lactic acid bacteria (LAB) have been extensively studied due to their bacteriocidal activities against food-borne pathogens and food spoilage bacteria (Drider et al. 2006). Among bacteriocins produced by LAB, the subclass IIa is defined as a group of antilisterial small, heat-stable, non-lanthionine-containing, and membrane-active peptides. Over the last two decades, major advances have been made on the genetic and molecular characterization, regulation of biosynthesis, and antimicrobial activity spectra of several class IIa bacteriocins (Ennahar et al. 2000; Drider et al. 2006).

Research on the production of bacteriocins has received a considerable attention due to the potential application of bacteriocins in the food industry as natural preservatives or in pharmacology as potential alternatives to antibiotics. However, bacteriocin yields with the native host are often low (Rodriguez et al. 2003). Maximum fermentation yields reported for the most studied bacteriocins, nisin, and pediocin, are in the range from 100 to 200 mg/l (Goulhen et al. 1999; Bertrand et al. 2001). Furthermore, the isolation and purification of class IIa bacteriocins from culture supernatant usually involve complex steps, are time-consuming, and result in very low protein yields (Guyonnet et al. 2000). To improve production and purification of bacteriocins, several heterologous expression systems have been developed and production of recombinant proteins has been studied in different host organisms, such as lactic acid bacteria, *Escherichia coli* (Rodriguez et al. 2003; Gibbs et al. 2004), and yeasts (Gutierrez et al. 2005b). Yields of

class IIa bacteriocins such as mesenterin Y105, piscicolin 126, and pediocin PA-1 could be increased during cultivation of recombinant host in shake flasks, although no culture optimization in controlled bioreactor fermentation was reported (Biet et al. 1998; Horn et al. 1999; Ingham et al. 2005).

Divercin V41, a class IIa bacteriocin produced by *Carnobacterium divergens* V41, was isolated from fish and characterized (Metivier et al. 1998; Pilet et al. 1995). A high anti-listeria activity of *C. divergens* V41 and divercin V41 was demonstrated in cold-smoked salmon (Richard et al. 2003). Recently, an efficient expression system was developed for the heterologous expression of divercin V41 (Richard et al. 2004). To express a recombinant divercin V41 (DvnRV41), a synthetic gene *dvnRV41* that encodes the mature divercin V41 peptide was constructed and over-expressed as a translational fusion protein with thioredoxin in pET-32b by using isopropyl β-D-thiogalactopyranoside (IPTG) inducible T7 RNA polymerase promoter in *E. coli* Origami (DE3) (pLysS) (Richard et al. 2004). The *dvnRV41* gene cloned into the expression vector allowed the expression of the fusion protein TRX-$(His)_6$-DvnV41.

The fusion protein TRX-$(His)_6$-DvnRV41 accumulated in cell cytoplasm in a soluble anti-*Listeria* active form and could be cleaved by an enzymatic hydrolysis or acid cleavage to release pure active DvnRV41. DvnRV41 could be purified using an immobilized metal-affinity chromatography column (IMAC). Such an expression and purification system enabled a yield of 23 mg of pure and highly active DvnRV41 per liter (Richard et al. 2004) during cultivations in shake flasks.

Although several heterologous expression systems have been developed, the potential of such systems to increase bacteriocin yields was not fully explored because cultivations of such heterologous systems was only performed in shake flasks or stirred reactors under uncontrolled conditions. In this study, we optimized the induction of T7 RNA polymerase promoter to increase the TRX-$(His)_6$-DvnRV41 fusion protein yield and tested optimized conditions for production of recombinant divercin V41 during batch and fed-batch cultures of *E. coli* Origami (DE3) (pLysS/pCR03).

Materials and methods

Bacterial strains

Escherichia coli K-12 strain Origami (DE3) (pLysS/pCR03) (Novagen, Madison, WI, USA) was used for the expression of recombinant divercin V41 as a fusion protein (TRX-$(His)_6$-$(Asp)_4$-Lys-Ala-Met-Asp-Pro-DvnRV41) (Richard et al. 2004), with *E. coli* K-12 strain Origami (DE3) (pLysS) as negative control. *Listeria innocua* F (Metivier et al. 1998) was used as a divercin V41 sensitive indicator organism. Cultures were stored in 50% glycerol stocks at −80°C.

Media and preculture preparation

Escherichia coli Origami (DE3) (pLysS/pCR03) and *E. coli* Origami (DE3) (pLysS) were grown in Terrific broth (TB) complex or M9 mineral medium (Sambrook and Russell 2001) supplemented with 30 μg/ml chloramphenicol. One liter of M9 mineral medium was supplemented with 20 ml of 20% (*w*/*v*) glucose, 200 ml 10% (*w*/*v*) casein peptone (Fluka, Buchs, Switzerland) solution and 1 ml of trace element solution US* (Panke et al. 1999). For *E. coli* Origami (DE3) (pLysS/pCR03) cultivation, the medium was also supplemented with 100 μg/ml ampicillin. *L. innocua* F was grown in Tryptone Soy Broth (TSB) (Oxoid, Hampshire, England). Preculture of *E. coli* strains were prepared by inoculating 100 ml media with 1 ml glycerol stock and incubating overnight at 37°C on a rotary shaker at 200 rpm. To prepare preculture of *L. innocua* F, 25 ml TSB medium was inoculated with 250 μl of glycerol stock then incubated at 30°C without shaking for 16 h.

Cultivation of *E. coli* Origami (DE3) (pLysS/pCR03) in shake-flasks

M9 mineral medium (100 ml) was inoculated with a preculture of *E. coli* Origami (DE3) (pLysS/pCR03) to an OD_{600} of 0.3 and incubated at 30°C on a rotary shaker at 200 rpm. The culture was induced with IPTG when the OD_{600} reached 1.0 and further incubated under the same conditions. Samples were taken every hour and cells were harvested by centrifugation and disrupted, fusion protein was cleaved, and antilisterial activity of the cleaved cytoplasmic soluble fraction (CSF) was measured as described below. Fusion protein in filtered CSF of the last sample after 7 h incubation was purified and quantified. After cleavage, the released free DvnRV41 was purified on His-trap chelating column and quantified as described below. For the determination of the effect of IPTG concentration on the production of TRX-$(His)_6$-DvnRV41, cultures in TB medium were induced with different IPTG concentrations (0, 0.05, 0.25, 0.5, 1.0, and 2.0 mM), then incubated for 3 h. The OD_{600} of the cultures was monitored. After incubation, the cells were disrupted and the proteins of the filtered CSF were separated via tricine SDS-PAGE as described below. Reported data are means for triplicates of independent experiments.

Batch cultures of *E. coli* Origami (DE3) (pLysS/pCR03)

Batch cultures of *E. coli* Origami (DE3) (pLysS/pCR03) were carried out in a stirred tank reactor (total volume of 3 l; Bioengineering, Wald, Switzerland), equipped with two Rushton turbine impellers and four baffles. The reactor was filled with 1,800 ml of M9 mineral medium and inoculated with 200 ml *E. coli* Origami (DE3) (pLysS/pCR03) preculture. Cultures were performed at 30°C with aeration at 2 l/min, pH was controlled at 7.4 by adding 5 M NaOH, and stirring set at 1,000 rpm. Silicon oil-based antifoam agent (Sigma Aldrich) was used to control foaming. Cultures were induced 5 h after the start of cultivation with addition of IPTG at a final concentration of 0.5 mM. Cell growth was monitored by optical density measurements, and samples were taken every hour. Cells were disrupted, fusion protein was cleaved, and antilisterial activity of the cleaved CSF was measured. The specific growth rate was calculated from biomass measurements by least-square fitting to linear parts of semilogarithmic plots of optical density at 600 nm vs time. Reported data are means for triplicates of independent experiments.

Fed-batch cultures of *E. coli* Origami (DE3) (pLysS/pCR03)

First, a batch cultivation of *E. coli* Origami (DE3) (pLysS/pCR03) was performed for 10 h after which stationary phase was reached. The medium was then supplemented with 2 ml chloramphenicol solution (30 mg/ml), 4 ml ampicillin solution (50 mg/ml), 4 ml of trace element solution US* and 20 g casein peptone. For fed-batch culture an aqueous solution containing 50% (*v*/*v*) glycerol and 1% (*w*/*v*) $MgSO_4$ was added at a rate of 6 ml per hour, and was increased to 15 ml per hour during cultivation to maintain the dissolved oxygen tension between 20 and 40% saturation. Cultures were induced 4 h after starting the feeding by adding IPTG to a final concentration of 0.5 mM. Sampling and analysis were done as for batch cultures.

Determination of cell dry weight of *E. coli* Origami (DE3) (pLysS/pCR03)

For cell dry weight determinations, 10 ml of culture sample was centrifuged for 15 min at 8,000×*g*. The supernatant was discarded and the pellet was resuspended in 1 ml distilled water. Resuspended samples were filtered under vacuum through a dried and pre-weighed 0.45 μm filter (type HVPL Millipore, Volketswil, Switzerland), dried in an oven at 80°C until constant weight, and accurately weighted. A correlation between cell dry weight and OD_{600} was determined with shake flask cultures, and used to calculate biomass concentration from OD_{600} during shake flask, batch, and fed-batch cultivations.

Protein analyses

Protein concentration was determined using the BCA protein assay reagent according to the supplier's protocol, using bovine serum albumin (Pierce, Rockford, IL, USA) was used as standard. Reported data are means for duplicate analyses. Proteins were separated under reducing conditions by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (Schagger and Vonjagow 1987). Proteins in Tricine SDS-PAGE (16.5% polyacrylamide) gels were stained with Coomassie blue R-250 (LKB, Bromma, Sweden). An ultra low-range marker (Bio-Rad, Reinach, Switzerland) was used as a molecular mass marker (26.625, 16.950, 14.437, 6.512, 3.496, and 1.423 kDa).

Purification of recombinant fusion protein and free DvnRV41

Cells were harvested by centrifugation (8,000×*g*, 6 min, 4°C). The cell pellet was resuspended in binding buffer containing 10 mM imidazol (BB10; pH 7.9) (Fluka, Buchs, Switzerland) to yield a cell concentration of 35 g cell dry weight per liter (g cdw/l). The cells were disrupted twice via French press (Sim Aminco, SLM instruments, Rochester, NY, USA.). The cytoplasmic soluble fraction (CSF) was separated by centrifugation (14,000×*g*, 15 min, 4°C), filtered (0.45 μm, Millipore, Volketswil, Switzerland) and then loaded directly onto a 1-ml nickel His-Trap chelating column (Amersham Biosciences, Uppsala, Sweden).

After loading, the column was successively washed with 10, 20, and 60 mM imidazole, pH 7.9. The TRX-$(His)_6$-DvnRV41 fusion protein was eluted with 2 ml of 500 mM imidazole (pH 7.9). To release DvnRV41 from its fusion partner, a tenfold diluted filtered CSF (2 ml) was adjusted to pH 1.3 with 5 N HCl. After incubation at 50°C for 24 h, the pH of the solution was increased to 7 with 2 M NaOH, and the antilisterial activity of the cleaved CSF was tested. The separation of DvnRV41 and TRX-$(His)_6$ from the fusion protein was achieved by a second immobilized metal-affinity chromatography step with the same conditions. The DvnRV41 was found in the flow through fraction and the noncleaved fusion protein and TRX-$(His)_6$ in the fraction eluted by 500 mM imidazole.

Antilisterial activity test

The antilisterial activity of cleaved CSF was determined with a turbidometric microplate bioassay (Turcotte et al. 2004). Wells of a sterile 96-well tissue culture plate with

flat bottom (BioConcept, Allschwil, Switzerland) were filled with 125 µl of TSB as diluting medium. The tenfold diluted cleaved CSF was diluted 100-fold with sterile high performance liquid chromatography (HPLC) grade water. After adding 125 µl of diluted sample in the first well, serial twofold dilutions were made. Finally, 50 µl of a preculture of the indicator strain *L. innocua* F diluted 1/1,000 in TSB was inoculated into each well. The OD_{590} of the wells was read before and after 13 h incubation at 30°C without shaking with a microplate reader (Bio-Tek, Witec AG, Littau, Switzerland). The activity expressed in arbitrary units per ml was determined using the formula: $(1{,}000/125)\times 2^n$, where n is the number of wells showing inhibition. Wells with inhibited growth were defined as wells with an OD_{590} less than half of the OD_{590} of the positive control with no added bacteriocin. A negative control well was filled with 175 µl TSB. Antilisterial activity was expressed as AU per liter. Reported data are means for duplicate analyses.

Statistical analysis

Univariate ANOVA was performed using the general linear model of SPSS 13.0 (SPSS, Chicago, IL, USA) at a level of significance of $p<0.05$. When statistically significant differences were shown, treatment means were compared using Tukey`s multiple comparison test. Bacteriocin activity data obtained with the serial twofold dilution test are discontinuous and therefore were not analyzed statistically. However, bacteriocin titers were highly reproducible for the repeated cultures and analyses and differed by no more than one well, corresponding to a twofold dilution.

Results

Effect of IPTG concentration

SDS-PAGE pattern of non-induced and induced cultures showed the presence of a band at 20 kDa (data not shown), characteristics of TRX-$(His)_6$-DvnRV41 fusion protein (Richard et al. 2004). The non-induced cells showed a specific growth rate of 0.47±0.01 h^{-1}, which was not significantly different for cells induced with 0.05 or 0.25 mM of IPTG (0.43±0.04 and 0.42±0.02 h^{-1}, respectively). Induction of cells with 0.5 mM of IPTG significantly decreased the specific growth rate (0.36±0.01 h^{-1}) compared to non-induced cells, but not significantly compared with cultures induced with 0.05 and 0.25 mM IPTG. However, induction of cells with 1 or 2 mM of IPTG yielded lower specific growth rates (0.24±0.01 and 0.23±0.03 h^{-1}, respectively) compared to all other tested conditions.

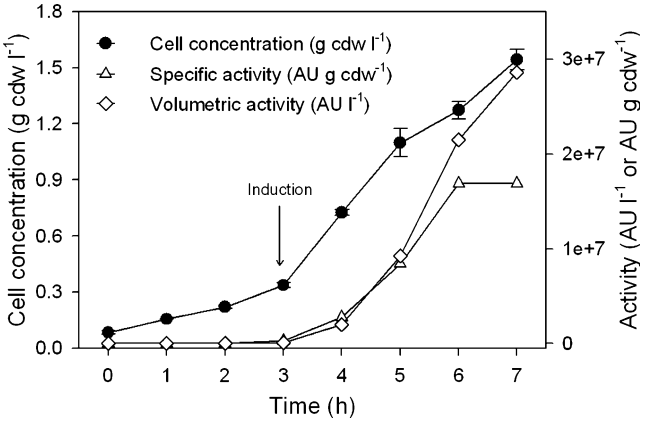

Fig. 1 Growth of *E. coli* Origami (DE3) (pLysS/pCR03) and antilisterial activity of cleaved cytoplasmic soluble fractions during cultivation at 37°C in shake flasks. Cells were induced with 0.5 mM IPTG when the OD_{600} reached 0.3

Cultivation of *E. coli* Origami (DE3) (pLysS/pCR03) in shake flasks

During cultivation of *E. coli* Origami (DE3) (pLysS/pCR03) in shake flasks, cell concentration increased from 0.08±0.01 g cdw/l to 1.54±0.06 g cdw/l in 7 h (Fig. 1). No specific antilisterial activity was detected before the induction during the first 3 h. After induction, volumetric bacteriocin activity increased until the end of the cultivation, reaching 2.8×10^7 AU/l after 7 h (Fig. 1). Specific activity increased after induction until 6 h incubation and then remained constant. The final concentrations of TRX-$(His)_6$-DvnRV41 fusion protein and free DvnRV41 were 74±5 and 18±1 mg/l, respectively.

Batch culture of *E. coli* Origami (DE3) (pLysS/pCR03)

Cell growth and specific and volumetric antilisterial activities during batch cultures of *E. coli* Origami (DE3)

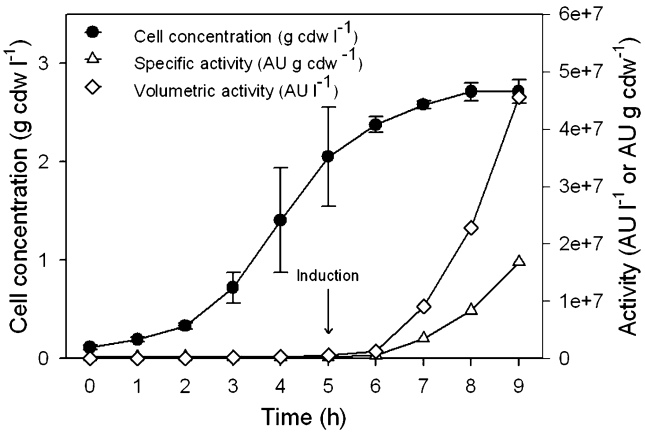

Fig. 2 Growth of *E. coli* Origami (DE3) (pLysS/pCR03) and antilisterial activity of cleaved cytoplasmic soluble fractions during a batch cultivation in M9 medium in a 2-l bioreactor. Cultures were induced with 0.5 mM IPTG 5 h after the start of the cultivation

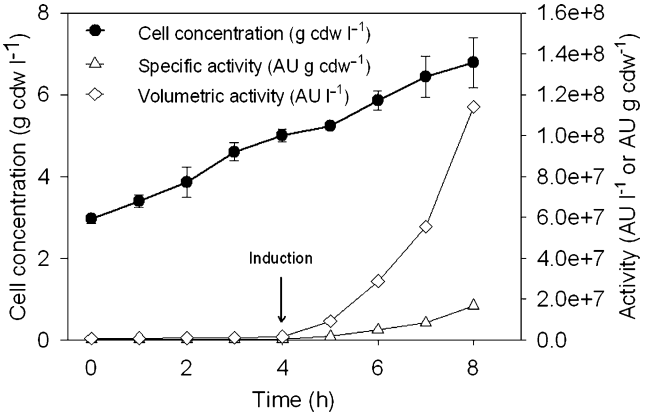

Fig. 3 Growth of *E. coli* Origami (DE3) (pLysS/pCR03) and antilisterial activity of cleaved cytoplasmic soluble fractions during a fed-batch cultivation in M9 medium in a 2-l bioreactor. After 10 h of batch cultivations (corresponding to time 0 h in the graph), the culture was fed with glycerol solution. Cells were induced with 0.5 mM IPTG 4 h after the start of the fed-batch

(pLysS/pCR03) are presented in Fig. 2. Before induction, cells grew to 1.5 g cdw/l after 4 h incubation with a specific growth rate of 0.46±0.02 h^{-1}. After induction (5 h), the specific growth rate decreased to 0.12±0.03 h^{-1} and cell concentration reached 2.7±0.1 g cdw/l after 9 h. Both specific and volumetric antilisterial activities increased after 5 h culture and reached 1.7 10^7 AU/g cdw and 4.6 × 10^7 AU/l, respectively at the end of the 9 h culture. At the end of batch cultures TRX-$(His)_6$-DvnRV41 fusion protein and free DvnRV41 reached high concentrations of 118±7 and 30±2 mg/l, respectively.

Fed-batch culture of *E. coli* Origami (DE3) (pLysS/pCR03)

After 10 h batch culture, the stationary growth phase was reached with a cell density of 2.9±0.1 g cdw/l. When feeding started cells began to grow again with a specific growth rate of 0.135±0.001 h^{-1} and reached to a cell density of 5.0±0.1 g cdw/l after 14 h (Fig. 3) when IPTG induction was started. Then the specific growth rate decreased to 0.08±0.02 h^{-1} and cell concentration reached 6.8±0.6 g cdw/l at the end of the 18 h culture. After cell induction, both specific and volumetric antilisterial activities increased and reached 1.7 × 10^7 AU/g cdw and 11.4 × 10^7 AU/l after 18 h, respectively. At the end of fed-batch culture, TRX-$(His)_6$-DvnRV41 fusion protein and free DvnRV41 concentrations reached 296±20 and 74±5 mg/l, respectively.

Discussion

Various promoter sequences have been characterized and used for the regulation of heterologous gene expression in *E. coli* (Goldstein and Doi 1995). The IPTG inducible *lac* expression system of *E. coli* is often used for producing recombinant proteins. Expression levels can be tuned by optimizing the induction via varying the inducer concentrations. Use of high concentrations of inducer to fully induce the promoter does not necessarily lead to maximal expression of a target protein due to metabolic burden and toxicity of inducers to the cells (Glick 1995). The T7 expression system, which relies on the lac promoter, has a relatively high basal expression in the absence of inducer (Weickert et al. 1996).

The low level expression of recombinant fusion protein in the non-induced culture could be due to the high activity of T7 polymerase, which can lead to a substantial

Table 1 Heterologous expression of class IIa bacteriocins

Bacteriocin	Host strain	Culture type	Bacteriocin concentration (mg/l)	Reference
Mesenterin Y105	*E. coli* DH5α (PFBYC02)	Shake flask	1	(Biet et al. 1998)
Pediocin PA-1	*L. lactis* IL1403	Shake flask[a]	2	(Horn et al. 1999)
Pediocin PA-1	*L. lactis* IL1403	Shake flask[a]	0.4	(Martinez et al. 2000)
Enterocin A	*L. lactis* IL1403	Shake flask[a]	0.027	(Martinez et al. 2000)
Piscicolin 126	*E. coli* AD494 (DE3)	Fed-batch[a,b]	26	(Gibbs et al. 2004)
Divercin V41	*E. coli* Origami (DE3)	Shake flask	23	(Richard et al. 2004)
Piscicolin 126	*E. coli* BL21 (DE3)	Shake flask	1.1	(Ingham et al. 2005)
Enterocin P	*E. coli* Tuner (DE3)	Shake flask[a]	0.006	(Gutierrez et al. 2005a)
Divercin V41	*E. coli* Origami (DE3)	Shake flask	18	This work
Divercin V41	*E. coli* Origami (DE3)	Batch	30	This work
Divercin V41	*E. coli* Origami (DE3)	Fed-batch	74	This work

[a] Cultivations were done in shake flasks or in bioreactors with only temperature control.
[b] Additional glucose was added to the culture after induction and at 2 h postinduction.

expression of target protein at small basal level (Studier 2005). Basal expression could also be due to the presence of an ingredient in the TB broth that mimics IPTG and deregulates the repression mechanism of the *lac* repressor protein (Delcarte et al. 2003).

The inducer concentration of 0.5 mM was chosen for batch and fed-batch cultures in bioreactor because it led to both high specific TRX-$(His)_6$-DvnRV41 fusion protein production and only a limited decrease of the specific growth rate compared with lower IPTG concentrations.

The highest bacteriocin yield during cultivation of heterologous expression systems in shake flasks was previously reported for *E. coli* Origami (DE3) (pLysS/pCR03) by Richard et al. (2004) (Table 1). We achieved slightly lower bacteriocin yields with same expression system in shake flask cultivations, which might be explained by different media. Gibbs et al. (2004) reported a bacteriocin yield of 26 mg piscicolin per liter obtained with repeated addition of glucose in fed-batch cultivation of heterologous expression system *E. coli* in a 2.4-l medium. In our study, high recombinant protein concentrations of 30±2 and 74±5 mg DvnRV41/l, were obtained with batch and fed-batch cultures of *E. coli* Origami (DE3) (pLysS/pCR03) in a 2-l controlled bioreactor, respectively. The increase of DvnRV41 production during fed-batch cultures correlated with increased cell production, which was more than threefold higher than that measured during shake flask cultures in this study and by Richard et al. (2004).

At the end of the fed-batch culture, cell concentration was not leveled off. Therefore, the production of DvnRV41 could be further increased by increasing the cultivation time. Development of heterologous expression systems for bacteriocin production using ABC transporters or sec-dependent secretion systems could further increase bacteriocin yield in controlled cultivation systems.

To our knowledge, this study is the first controlled fed-batch application of heterologous expression of bacteriocin production. Our results clearly showed that bacteriocin production can be increased significantly with different production strategies such as batch and fed-batch cultivations. High yield production of recombinant bacteriocins may facilitate characterization of the molecules and of their mode of action, and may broaden the application of bacteriocins in industry. Although applications of recombinant bacteriocins in food is limited by current regulations, such compounds have potential in clinical applications, especially as new antimicrobial agents active against pathogens with multiple antibiotic resistances (Valsesia et al. 2007).

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