

Metabolic engineering of *Lactococcus lactis* influence of the overproduction of lipase enzyme

Mohammad Raftari¹, Sobhan Ghafourian² and Fatimah Abu Bakar^{1*}

¹ Faculty of Food Science and Technology, Universiti Putra Malaysia, UPM 43400, Serdang, Selangor, Malaysia

² Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran

Received 21 January 2013; accepted for publication 16 July 2013; first published online 24 September 2013

The dairy industry uses lipase extensively for hydrolysis of milk fat. Lipase is used in the modification of the fatty acid chain length, to enhance the flavours of various chesses. Therefore finding the unlimited source of lipase is a concern of dairy industry. Due to the importance of lipase, this study was an attempt to express the lipase from *Burkholderia cepacia* in *Lactococcus lactis*. To achieve this, a gene associated with lipase transport was amplified and subcloned in inducible pNZ8148 vector, and subsequently transformed into *Lc. lactis* NZ9000. The enzyme assay as well as SDS-PAGE and western blotting were carried out to analysis the recombinant lipase expression. Nucleotide sequencing of the DNA insert from the clone revealed that the lipase activity corresponded to an open reading frame consisting of 1092 bp coding for a 37.5-kDa size protein. Blue colour colonies on Nile blue sulphate agar and sharp band on 37.5-kD size on SDS-PAGE and western blotting results confirm the successful expression of lipase by *Lc. lactis*. The protein assay also showed high expression, approximately 152.2 µg/ml.h, of lipase by recombinant *Lc. lactis*. The results indicate that *Lc. lactis* has high potential to overproduce the recombinant lipase which can be used commercially for industrially purposes.

Keywords: *Lactococcus lactis*, lipase, overproduction.

Recent years have seen an increasing interest in the genetics and physiology of lactic acid bacteria because of their importance for major economic activities, primarily their use in a variety of dairy fermentations (Mierau & Kleerebezem, 2005).

Lactococcus lactis, the model lactic acid bacterium (LAB), is a food grade Gram-positive bacterium, which is well-characterised and 'generally recognised as safe' (GRAS). Many genetic tools have been developed and its complete genome was sequenced (Wessels et al. 2004). Protein secretion by this GRAS bacterium would allow production directly in a food product and thus an interaction between the secreted protein (enzyme or antigen) and the environment (the food product itself or the digestive tract). *Lc. lactis* can be considered a good candidate for heterologous protein secretion for at least two reasons: (i) to date, relatively few proteins are known to be secreted by *Lc. lactis* (ii) secreted proteins are prone most of the time to extracellular degradation (Wu et al. 1998; Lee et al. 2000).

High-level production of heterologous proteins in *Lc. lactis* has been obtained using lactococcal constitutive or inducible promoters (Kuipers et al. 1997; de Vos, 1999). One of the developed expression systems for *Lc. lactis* is the Nisin Controlled gene Expression system (NICE). This system has become one of the most successful and widely used tools for regulated gene expression in Gram-positive bacteria (Mierau & Kleerebezem, 2005).

The NICE system has been used to express genes of various different backgrounds (Gram-positive, Gram-negative and eukaryotic) to study metabolic and enzyme function and to produce larger amounts of an enzyme for food, medical or technical applications (de Ruyter et al. 1996).

Lipases constitute the most important group of biocatalysts for biotechnological applications (Benjamin & Pandey, 1998). Lipases have been isolated from many species of plants, animals, bacteria, fungi and yeast. The enzymes from microorganisms are used in various industries such as dairy, food, detergents, textile, pharmaceutical, cosmetic and biodiesel industries, and in synthesis of fine chemicals, agrochemicals and new polymeric materials (Saxena et al. 1999). Lipases are extensively used in the dairy industry for hydrolysis of milk fat. The dairy industry uses lipases in the

*For correspondence; e-mail: fatim@putra.upm.edu.my

Table 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant properties	Source
Bacterial strain		
<i>Bur. cepacia</i> 21808	ATCC	
<i>Esch. coli</i> TOP10	F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
<i>Lc. lactis</i> NZ9000	MG1363 pepN::nisRK	MoBiTec
Plasmids		
PCRBlunt Vector	Amp ^r	Fermentas
pNZ8148	Cm ^r inducible expression vector carrying PnisA	MoBiTec

modification of fatty acid chain lengths, to enhance the flavours of various cheeses. Current applications also include the acceleration of cheese ripening and the lipolysis of butter, fat and cream (Jaeger & Eggert, 2002). The free fatty acids generated by the action of lipases on milk fat endow many dairy products, particularly soft cheeses, with their specific flavour characteristics (Sharma et al. 2001).

Burkholderia cepacia lipase formerly known as *Pseudomonas cepacia* lipase has been shown to be a useful enzyme which catalyses a broad range of different reactions in water and non-polar solvents under mild conditions. *Bur. cepacia* lipase is a highly selective catalyst for a broad range of substrates (Kazlauskas & Weber, 1998), including the kinetic resolution of racemic mixtures of secondary alcohols by hydrolysis in water or esterification in organic solvents (Schmid & Verger, 1998; Bornscheuer & Kazlauskas, 1999; Jaeger & Eggert, 2002).

Due to the importance of lipase enzyme and many advantages of *Lc. lactis* as a cell factory as well as unique characteristics of NICE system, this study was an attempt to clone and highly express the lipase from *Bur. cepacia* in *Lc. lactis* using NICE protein expression system.

Material and methods

Bacterial strains, media and growth condition

The bacterial strains and plasmids used are listed in Table 1. *Lc. lactis* was grown statically in M17 medium (Oxoid, USA) at 30 °C in tightly capped flasks supplemented with 0.5% glucose. *Escherichia coli* was grown in Luria-Bertani (LB) medium (Oxoid, USA) at 37 °C. Chloramphenicol was used at a concentration of 10 µg/ml, for *Lc. lactis* and ampicillin was used at concentration of 100 µg/ml for *Esch. coli* when needed. Nisin powder (ICN; 2.5% nisin content) was used at concentrations of 1, 5, 10 and 15 ng/ml.

DNA manipulation

Chromosomal DNA of *Bur. cepacia* was obtained using miniprep DNA extraction Kit (GenAll, Korea) according to the manufacturer's instructions. The presence and quality of genomic DNA was confirmed by agarose gel

electrophoresis. The optical density (OD) ratio at 260 and 280 nm (OD₂₆₀/OD₂₈₀) was calculated to estimate the purity of extracted DNA. Plasmid DNA was isolated using plasmid extraction miniprep spin kit (Fermentas) according to the manufacturer's instructions.

Synthesis and sequencing lipase gene

Lipase gene (LipA) from *Bur. cepacia* was amplified by PCR using a pair of primers designed according to the lipase nucleotide sequences deposited in GeneBank under accession number (M58494). The PCR product was cloned into *Esch. coli* TOP10 cells using the pCR Blunt vector (Fermentas).

The recombinant plasmid was sequenced by Sigma Company (Singapore). The results of DNA sequencing were then run in Chromas Lite program to analyse the similarity of sequenced gene in GenBank library. The comparison of the DNA sequence was carried out using the database of the National Centre of Biotechnology (<http://www.ncbi.nih.gov>). Meanwhile, the analysis of the lipase gene was achieved with Biology Workbench (<http://www.biology.ncsa.sdsc.edu>) and ExPASy (Expert Protein Analysis System) (<http://www.expasy.org.tools>).

Cloning and expression of lipase gene

A set of primers were designed based on the sequence of *Bur. cepacia* (Forward: 5'-ATGGCCAAATCGATGCGTTCC-3' and Reverse: 5'-TTACACGCCCGGAGCTTCA-3'). Amplification process was carried out in a reaction mixture containing 50–100 ng DNA template, 30 pmol each forward and reverse primers, 0.2 mM dNTP mix, 2 mM MgCl₂, 2 U *Taq* DNA polymerase (Fermentas; Lithuania), 10×PCR buffer with the following PCR conditions: an initial denaturation step at 94 °C for 4 min, 35 cycles at 94 °C for 1 min, annealing at 64 °C for 50 s, and extension at 72 °C for 2 min, except for the final extension of 10 min, and preservation at 4 °C.

The PCR product was directly cloned into the pCR-Blunt vector (Fermentas) according to the manufacturer's instructions and transformed into *Esch. coli* Top 10 competent cells. The positive clones were obtained on the LB agar plate contains 100 µg/ml ampicillin after 24 h at 37 °C. The positive clones were confirmed using colony PCR as well

as plasmid extraction following to the run on gel electrophoresis.

Expression of lipase gene in *Lactococcus lactis*

The PCR-Blunt vector used as template to amplify the lipase gene using following primers; Forward 5'-GCCTGCAGCATC-ACCATCACCATCACGGGATGGCCAAATCGATGCGTTCC-3' and Reverse 5'-AAGCTTTTACACGCCCGCGAGCTTCA-3', to generate a product that would encode a LipA recombinant protein with a six His-tag at the N-terminus. In the forward primer, nucleotides representing the six histidine residues (italicised) are located downstream of the incorporated *Pst*I restriction site (underlined); in the reverse primer, a *Hind*III restriction site (underlined) was incorporated downstream of the LipA sequence.

PCR was optimised and performed with initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 50 s, and 72 °C for 2 min. Amplification was ended at 72 °C for 10 min. The corresponding 1092 bp PCR product was purified and digested with both *Pst*I and *Hind*III and ligated into the corresponding digested sites in pNZ8148, producing the recombinant plasmid pNZ-LipA.

Competent *Lc. lactis* NZ9000 cells were then electro-transformed with the recombinant plasmids (Eppendorf, USA) at 2000 V for 5 ms, spread on M17 agar contains 0.5% glucose and 10 µg/ml Chloramphenicol incubated at 30 °C for 24 h (Holo & Nes, 1989). Transformants harbouring the recombinant plasmids were verified through enzymatic digestion; PCR and sequencing verified the correct lipase gene sequence, orientation, and frame.

Protein analysis

Overnight culture of recombinant *Lc. lactis* (pNZ-lipA) was diluted 100-folds in fresh M17-glucose-chloramphenicol and grown at 30 °C until an OD_{600 nm} value of 0.5–1. Expression of lipase was induced with different concentration of nisin 1, 5, 10 and 15 ng/ml at 30 °C. Preparation of protein was carried out according to Schmidtke et al. (1997) with some modifications. Bacterial cells were collected by two-times centrifugation at 4000 g for 5 min followed by washing and resuspension in phosphate buffered saline (PBS) containing 1% (w/v) sterile, acid-washed glass-beads (Merck, Germany). The cells were disrupted by vortexing for 1 min at maximum speed, and then chilled on ice for 2 min, repeating this process 8 times. The suspension was centrifuged at 4000 g for 20 min at 4 °C and the resulting supernatant was transferred into a separate tube. Both cellular supernatant and cell pellet were treated with 2% SDS detergent at 37 °C for 30 min before being centrifuged at 20 000 g for 30 min at 4 °C.

SDS-PAGE and western blotting

SDS-PAGE was carried out according to the protocol of Laemmli (1970). The protein preparations were further

subjected to boiling at 100 °C for 5 min in SDS and dithiothreitol (DTT) before being analysed on a 12% (w/v) denaturing polyacrylamide gel. SDS-PAGE resolved polypeptides were transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry electroblotter (BioRad, USA) at 64 mA for 45 min.

Lipase activity assay

Liberated free fatty acids were determined by calorimetric method using olive oil as a substrate (Rahman et al. 2010). An equal volume of olive oil (Merck, Germany) and 50 mM phosphate buffer (pH 7) was mixed to prepare the emulsion. One millilitre of enzyme was added to 2.5 ml of the emulsion plus 20 µl 0.02 M CaCl₂ and was shaken at 200 rpm for 30 min at 37 °C. One millilitre of 6 M HCl and 5 ml isooctane were then added to stop the enzyme reaction. This was followed by a vigorous mixing for 30 s with a vortex mixer. Four millilitres of the upper isooctane layer was transferred to a test tube containing 1 ml copper reagent. The copper reagent was prepared by 5% (w/v) copper (II) acetate-1-hydrate and the pH was adjusted to 6.1 with pyridine. The absorbance of the upper layer which contained the liberated fatty acids was read at 715 nm. Lipase activity was determined by measuring the amount of free fatty acid released by referring to the standard curves of free fatty acids. One unit of lipase activity was defined as the rate of 1 µmol of fatty acid released per minute.

Results

Cloning and sequencing of the lipase gene

PCR fragments of the expected size (1092 bp) were obtained using degenerated primers, cloned and sequenced (Fig. 1). Nucleotide sequence analysis revealed an open reading frame (ORF) starting at bp 1 with a ATG codon and ending at AAT at bp 1092, thereby encoding a protein of 364 amino acids. Lipases are known to have an active-site consensus sequence Gly-X-Ser-X-Gly and to form a catalytic triad consisting of Ser, Asp and His residues. The lipase had the Gly-X-Ser-X-Gly sequence at positions 106–110, and Ser, Asp and His residues at 208, 315 and 278, respectively. In addition, two Asp residues, known Ca⁺²-binding sites, were found at positions 240 and 279, and two Cys residues forming a disulphide bond at positions 210 and 261 were also conserved.

Expression of lipase in *Lc. lactis*

The lipase gene was successfully cloned and expressed in pNZ8148, an expression vector carrying nisin inducible nisA promoter, by *Lc. lactis* NZ9000 as host. To achieve this the lipase gene was inserted into pNZ8148 between *Pst*I and *Hind*III restriction sites, then the recombinant vector (pNZ-lipA) was transformed into *Lc. lactis* NZ9000 by

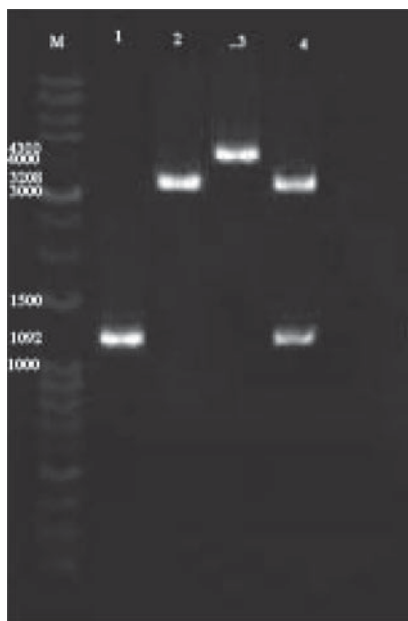


Fig. 1. Agarose gel electrophoresis results Gene amplification and cloned. (M) Marker, (1) PCR Amplification of lipase gene, (2) single digested pNZ8148, (3) single digested cloned pNZ-LipA, (4) Double digested pNZ-LipA.

electroporation method. The recombinant *Lc. lactis* was cultured on M17-Glu-Cm and incubated at 30 °C for 24 h.

To detect the lipase expression of recombinant *Lc. lactis* containing pNZ8148, *Lc. lactis* containing pNZ-lipA were cultured on the Nile Blue Sulphate agar, and incubated at 30 °C for 24 h. Screening the results showed no lipase expression in cultured plates with *Lc. lactis*-pNZ8148 (no blue colour colonies) while the dark blue colonies was observed in plate containing *Lc. lactis*-pNZ-lipA (results not shown). These primary screenings indicate the expression of lipase by recombinant *Lc. lactis*.

Expression of lipase in *Lc. lactis* was analysed using SDS-PAGE and western blotting techniques. Expression optimisations were carried out with 1, 5, 10 and 20 ng/ml nisin while, the highest protein expression was detected for 10 ng/ml nisin. In the protein extracts comparing induced and uninduced cultures for pNZ-lipA as well as the negative control (clones harbouring empty pNZ8148 plasmid), the SDS-PAGE results showed distinct bands corresponding to an expected protein size of 37.5 kDa which was predicted to be exclusively visible in the induced cultures (Fig. 2). Also, subsequent analysis by western blotting showed a clear expected band on 37.5 kDa which was only present in the induced cultures but not in the uninduced cultures or the negative control (Fig. 3). The results confirm successful expression of recombinant protein in *Lc. lactis*. Optimisation of the induction conditions showed that lipase expression can be induced using 1–15 ng/ml nisin with highest expression at 10 ng/ml nisin.

Functionality of the recombinant lipase of purified recombinant protein extracts was analysed by enzymatic

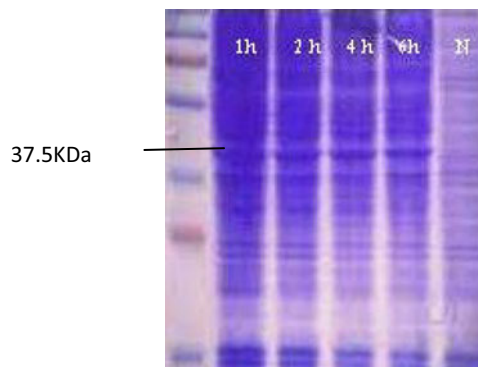


Fig. 2. SDS-PAGE results of lipase produced by *Lc. lactis* cells harbouring LipA. The highest protein expression was detected after 4 h of induction with 10 ng/ml nisin.

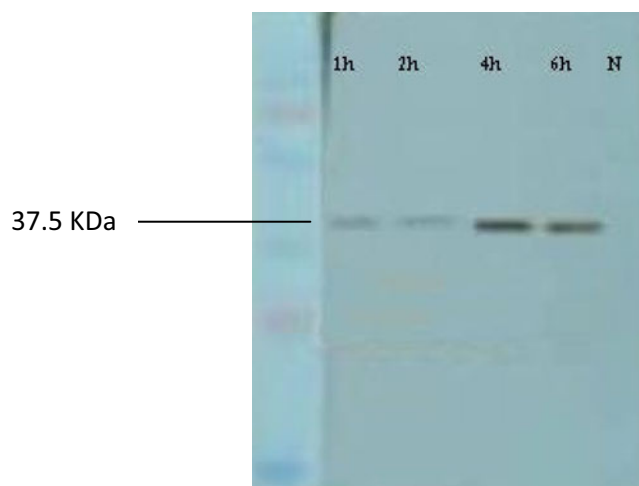


Fig. 3. Western Blotting results of lipase produced by *Lc. lactis* cells harbouring LipA. The highest protein expression was detected after 4 h of induction with 10 ng/ml nisin.

assay. The protein assay showed high lipase production by recombinant *Lc. lactis* around 152.2 µg/ml.h. The specific lipolytic activity of the recombinant lipase was found 180 U/ml which was 34 fold higher than the native enzyme.

Discussion

To date, many expression systems have been developed to produce recombinant proteins for various biotechnological applications. Among prokaryotic systems, the highest protein levels are obtained using *Esch. coli* as the cell factory (Jana & Deb, 2005; Raftari et al. 2012). However, in *Esch. coli*, the most commonly used production strategies are intracellular (in the periplasm or even in the cytoplasm), and involve expensive and often problematic downstream purification processes. In contrast, heterologous proteins produced in Gram positive bacterial hosts can be easily

secreted into the medium, thus facilitating their purification (Morello et al. 2008; Raftari et al. 2013).

Gram-positive bacterium having a GRAS status, *Lc. lactis*, is becoming an attractive alternative for heterologous protein secretion because of a number of properties which make it suitable for large scale protein expression; no inclusion bodies and endotoxin formation (Miyoshi et al. 2002; Nouaille et al. 2003; Peterbauer et al. 2011), simple fermentation procedure which make direct scale up from 1 to 1000 litres possible, self-cloning and food grade plasmid selection mechanism (Mierau et al. 2005a; Pinto et al. 2011), and finally being food grade (Konings et al. 2000; Nouaille et al. 2003; Peterbauer et al. 2011). *Lc. lactis* has been studied for the last 2 decades: its metabolism is relatively simple and well known, and the genome information of at least five strains of *Lc. lactis* were described and available (Bolotin et al. 2001; Klaenhammer et al. 2002). The comprehensive experience and knowledge gained has led to the application of *Lc. lactis* in areas other than in food production. It is used as a cell factory for pilot production of pharmaceutical products, production of human cytokines and other therapeutic agents for in situ treatments, expression of bacterial and viral antigens for safe vaccination via mucosal immunisation (Hols et al. 1999; Hugenholz et al. 2002; Nouaille et al. 2003; Hanniffy et al. 2004; Leroy & Devuyt, 2004; Mierau et al. 2005a).

The NICE system (de Ruyter et al. 1996; Kuipers et al. 1997, 1998) derives from the *nis* (*nisABTCIPRKEFG*) operon present in some *Lc. lactis* strains, which is involved in the biosynthesis of the antimicrobial peptide nisin (Kuipers et al. 1995). The NICE comprises the regulatory elements of the *nis* operon: *PnisA*, the nisin-inducible promoter (cloned into several expression vectors), and *nisRK*, the regulator-sensor 2-component system (either carried by compatible plasmids or integrated in the chromosome). The NICE system has been extensively used to produce proteins in *Lc. lactis* (Kunji et al. 2003; Le Loir et al. 2005; Mierau & Kleerebezem, 2005). It offers numerous advantages: (1) easy use, (2) tightly controlled and efficiently induced expression leading to high protein yields (Mierau & Kleerebezem, 2005) and (3) large-scale production process, as shown for *Staphylococcus simulans* lysostaphin (Mierau et al. 2005a, b).

In this study, we succeeded in overproduction of lipase from *Bur. cepacia* in *Lc. lactis* using NICE system. For achieving that the complete lipase gene according to sequence deposited in GeneBank, was cloned and expressed in *Lc. lactis* NZ9000 using pNZ8148 inducible vector. The lipase gene encoding an extracellular lipase cloned from the meta-genomic library was composed of 1092 bp in length. The predicted protein consisted of 364 amino acids with a calculated molecular mass of 37.5-kDa. Nucleotide sequence alignment showed that lipase gene has 100% identity with *Bur. cepaciae* lipase and the lowest identity was observed with *Vibrio vulnificus* lipase. In previous study (Raftari et al. 2012) we sub-cloned and expressed the lipase in *Esch. coli*. The results indicated

expression of recombinant lipase by *Esch. coli* but comparing the results showed that *Lc. lactis* has ability to produce higher level of recombinant lipase in shorter period, while extracellular expression by *Lc. lactis* could also reduce the purification cost and time as well as being safe.

Sequence analysis also showed that there is only one cysteine in the lipase protein, which is similar to other reported lipase (Steele & Streit, 2005). Cysteines are often involved in the formation of disulphide bonds in proteins, and proteins without cysteines are generally more flexible because of the lack of disulphide bonds (Li et al. 1995). This characteristic is important for protein secretion of extracellular bacterial proteins, which also readily allows the conformational change that accompanies interfacial activation.

In conclusion the NICE system was found to be a strong and practical protein expression system in *Lc. lactis* for production recombinant lipase enzyme that can be used for commercial purposes.

The authors are thankful to Ministry of Higher Education, Malaysia for financial support.

References

- Benjamin S & Pandey A 1998 Candida rugosa lipases: molecular biology and versatility in biotechnology. *Yeast* **14** 1069–1087
- Bolotin A, Wincker P, Mauger S, Jaillon O, Malarome K, Weissenbach J, Ehrlich SD & Sorokin A 2001 The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *Lactis* IL1403. *Genome Research* **11** 731–753
- Bornscheuer UT & Kazlauskas RJ 1999 *Hydrolases in Organic Synthesis: Regioand Stereoselective Biotransformations*. Weinheim: Wiley-VCH
- de Ruyter PG, Kuipers OP & de Vos WM 1996 Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Applied and Environmental Microbiology* **62** 3662–3667
- de Vos WM 1999 Gene expression systems for lactic acid bacteria. *Current Microbiology* **2** 289–295
- Hanniffy S, Wiedermann U, Repa A, Mercenier A, Daniel C, Fioramonti J, Tlaskolova H, Kozakova H, Israelsen H, Madsen S, Vrang A, Hols P, Delcour J, Bron P, Kleerebezem M & Wells J 2004 Potential and opportunities for use of recombinant lactic acid bacteria in human health. *Advances in Applied Microbiology* **56** 1–64
- Holo H & Nes IF 1989 High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Applied and Environmental Microbiology* **55** 3119–3123
- Hols P, Kleerebezem M, Schanck AN, Ferain T, Hugenholz J, Delcour J & de Vos WM 1999 Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. *Nature Biotechnology* **17** 588–592
- Hugenholz J, Kleerebezem M, Starrenburg M, Delcour J, de Vos W & Hols P 2002 *Lactococcus lactis* as a cell factory for high-level diacetyl production. *Applied and Environmental Microbiology* **66** 4112–4114
- Jaeger KE & Eggert T 2002 Lipases for biotechnology. *Current Opinion in Biotechnology* **13** 390–397
- Jana S & Deb JK 2005 Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Applied Microbiology and Biotechnology* **67** 289–298
- Kazlauskas RJ and Weber HK 1998 Improving hydrolases for organic synthesis. *Current Opinion in Chemical Biology* **2** 121–126
- Klaenhammer T, Altermann E, Arigoni F, Bolotin A, Breidt F, Broadbent J, Cano R, Chaillou S, Deutscher J, Gasson M, van de Guchte M, Guzzo J,

- Hartke A, Hawkins T, Hols P, Hutkins R, Kleerebezem M, Kok J, Kuipers O, Lubbers M, Maguin E, McKay L, Mills D, Nauta A, Overbeek R, Pel H, Pridmore D, Saier M, van Sinderen D, Sorokin A, Steele J, O'Sullivan D, de Vos W, Weimer B, Zagorec M & Siezen R 2002 Discovering lactic acid bacteria by genomics. *Antonie Van Leeuwenhoek* **82** 29–58
- Konings WN, Kok J, Kuipers OP & Poolman B 2000 Lactic acid bacteria: the bugs of the new millennium. *Current Opinion in Microbiology* **3** 276–282
- Kuipers OP, Rollema HS, Siezen RJ & de Vos WM 1995 Lactococcal expression systems for protein engineering of nisin. *Developments in Biological Standardization* **85** 605–613
- Kuipers OP, de Ruyter PG, Kleerebezem M & de Vos WM 1997 Controlled overproduction of protein by lactic acid bacteria. *Trends in Biotechnology* **15** 135–140
- Kuipers OP, de Ruyter PG, Kleerebezem M & de Vos WM 1998 Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology* **64** 15–21
- Kunji ER, Slotboom DJ & Poolman B 2003 *Lactococcus lactis* as host for overproduction of functional membrane proteins. *Biochimica et Biophysica Acta* **1610** 97–108
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685
- Lee SJ, Kim DM, Bae KH, Byun SM & Chung JH 2000 Enhancement of secretion and extracellular stability of staphylokinase in *Bacillus subtilis* by wprA gene disruption. *Applied and Environmental Microbiology* **66** 476–480
- Le Loir Y, Azevedo V, Oliveira SC, Freitas DA, Miyoshi A, Bermudez-Humaran LG, Nouaille S, Ribeiro LA, Leclercq S, Gabriel JE, Guimaraes VD, Oliveira MN, Charlier C, Gautier M & Langella P 2005 Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. *Microbial Cell Factories* **4** 2
- Leroy F & Devuyst L 2004 Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology* **15** 67–78
- Li XY, Tetling SS, Winkler UK, Jaeger KE & Benedik MJ 1995 Gene cloning, sequence analysis, purification, and secretion by *Escherichia coli* of an extracellular lipase from *Serratia marcescens*. *Applied and Environmental Microbiology* **61** 2674–2680
- Mierau I & Kleerebezem M 2005 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. *Applied Microbiology and Biotechnology* **68** 705–717
- Mierau I, Leij P, van Swam I, Blommestein B, Floris E, Mond J & Smid EJ 2005a Industrial-scale production and purification of a heterologous protein in *Lactococcus lactis* using the nisin-controlled gene expression system NICE: the case of lysostaphin. *Microbial Cell Factories* **4** 15
- Mierau I, Olieman K, Mond J & Smid EJ 2005b Optimization of the *Lactococcus lactis* nisin-controlled gene expression system NICE for industrial applications. *Microbial Cell Factories* **4** 16
- Miyoshi A, Poquet I, Azevedo V, Commissaire J, Bermudez-Humaran L, Domakova E, Leloir Y, Oliveira SC, Gruss A & Langella P 2002 Controlled production of stable heterologous proteins in *Lactococcus lactis*. *Applied and Environmental Microbiology* **68** 3141–3146
- Morello E, Bermúdez-Humarán LG, Llull D, Solé V, Miraglio N, Langella P & Poquet I 2008 *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. *Journal of Molecular Microbiology and Biotechnology* **14** 48–58
- Nouaille S, Ribeiro LA, Miyoshi A, Pontes D, Le Loir Y, Oliveira SC, Langella P & Azevedo V 2003 Heterologous protein production and delivery systems for *Lactococcus lactis*. *Genetic and Molecular Research* **2** 102–111
- Peterbauer C, Masischberger T & Haltrich D 2011 Food-grade expression in lactic acid bacteria. *Biotechnology Journal* **6** 1147–1161
- Pinto JPC, Kuipers OP, Marreddy RKR, Poolman B & Kok J 2011 Efficient overproduction of membrane proteins in *Lactococcus lactis* requires the cell envelope stress sensor/regulator couple CesSR. *PLOS ONE Journal* **6** 1–14
- Raftari M, Ghafourian S, Sadeghifard N, Sekawi Z, Saari N & Abu Bakar F 2012 Overexpression of recombinant lipase from *Burkholderia cepacia* in *Escherichia coli*. *European Journal of Inflammation* **10** 365–369
- Raftari M, Ghafourian S & Abu Bakar F 2013 Cloning and overexpression of extracellular elastase from *Pseudomonas aeruginosa*. *European Journal of Inflammation* **11** 55–60
- Rahman R, Kamarudin N, Yunus J, Salleh A & Basri M 2010 Expression of an organic solvent stable lipase from *Staphylococcus epidermidis* AT2. *International Journal of Molecular Sciences* **11** 3195–3208
- Saxena RK, Ghosh PK, Gupta R, Davidson WS, Bradoo S & Gulati R 1999 Microbial lipases: potential biocatalysts for the future industry. *Current Science* **77** 101–115
- Schmid RD & Verger R 1998 Lipases: interfacial enzymes with attractive applications. *Angewandte Chemie, International Edition* **37** 1609–1633
- Schmidtke G, Schmidt M & Kloetzel PM 1997 Maturation of mammalian 20 S proteasome: purification and characterization of 13 S and 16 S proteasome precursor complexes. *Journal of Molecular Biology* **268** 95–106
- Sharma R, Chisti Y & Banerjee UC 2001 Production, purification, characterization and applications of lipases. *Biotechnology Advances* **19** 627–662
- Steele HL and Streit WR 2005 Metagenomics: advances in ecology and biotechnology. *FEMS Microbiology Letters* **247** 105–111
- Wessels S, Axelsson L, Bech Hansen E, De Vuyst L, Låulund S, Låhtenmäki L & Lindgren S 2004 The lactic acid bacteria, the food chain, and their regulation. *Trends in Food Science and Technology* **15** 498–505
- Wu SC, Ye R, Wu XC, Ng SC & Wong SL 1998 Enhanced secretory production of a single chain antibody fragment from *Bacillus subtilis* by coproduction of molecular chaperones. *Journal of Bacteriology* **180** 2830–2835