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ORIGINAL ARTICLE

Factors Affecting Inducible Expression of Outer Membrane Protein A (OmpA) of *Shigella dysenteriae* Type-1 in *Lactococcus lactis* Using Nisin Inducible Controlled Expression (NICE)

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Abstract Potential use of *Lactococcus lactis* (*L. lactis*) as a heterologous protein expression host as well as for delivery of multiple therapeutic proteins has been investigated extensively using Nisin Inducible Controlled Expression (NICE) system. Optimum inducible expression of heterologous protein by NICE system in *L. lactis* depends on multiple factors. To study the unexplored role of factors affecting heterologous protein expression in *L. lactis* using NICE, the present study outlines the optimization of various key parameters such as inducer concentration, host's proteases and precipitating agent using Outer membrane protein A (OmpA). For efficient expression and secretion of OmpA, pSEC:OmpA vector was successfully constructed. To circumvent the troubles encountered during detection of expressed OmpA, the precipitating agent was switched from TCA to methanol. Nevertheless, detection was achieved accompanied by degraded protein products. Speculating the accountability of observed degradation at higher inducer concentration, different nisin concentrations were evaluated. Lower nisin concentrations were found desirable for optimum expression of OmpA. Consistently observed degradation was eliminated by incorporation of protease inhibitor cocktail which inhibits intracellular proteases and expression in VEL1153 (NZ9000 *ΔhtrA*) strain which inhibits extracellular protease leading to optimum expression of OmpA. Versatility and complexity of NICE system in *L. lactis* requires fine-tuning of target protein specific parameters for optimum expression.

Keywords Degradation · *Lactococcus lactis* · NICE · Nisin · Processing · Proteases

Introduction

Lactococcus lactis (*L. lactis*), generally regarded as safe (GRAS) microorganism produces myriad beneficial effects on human health and stimulates immune system when used as an antigen delivery vehicle. There has been recent efforts in developing *L. lactis* as a host for production of heterologous proteins of medical and technological interest [1]. Several inducible expression systems have been developed for expression of heterologous proteins. NICE is well documented and preferred expression system for *L. lactis*. NICE comprises of PnisA promoter and *nisRK* regulatory genes, wherein bacteriocin nisin acts as an inducer. *L. lactis* NZ9000 and its derivatives are widely used with NICE system having *nisRK* gene integrated into the chromosome of *L. lactis* MG1363 [2].

Usp45 is commonly used signal peptide in NZ9000 and its derivatives, for extracellular secretion of proteins [3]. Using NICE as an inducible expression system, several

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proteins such as IP-10, β-glucoronidase, Aminopeptidase N, NADPH oxidase 4 and various others have been expressed in *L. lactis* [2, 4].

We have expressed the outer membrane protein A (OmpA) of *Shigella dysenteriae* type-1 as a model protein in *L. lactis* using NICE. Although, NICE system is considered to be the most suitable for recombinant membrane protein expression [5], optimization of multiple factors for efficient expression is required. The role of variable factors in optimal expression of heterologous protein using NICE in *L. lactis* has not gained much attention. Thus, our study focuses on the variable factors involved in NICE using *L. lactis.*

The variants which were found to play an important role in protein expression include concentration of inducer, protein precipitating agent and effect of host proteases.

In *L. lactis* NZ9000, host proteases are present intracellularly as well as extracelluarly. A unique protease, High-temperature requirement A (HtrA), found in the extracellular matrix of *L. lactis,* is known to degrade the secreted protein [1]. Hence, we evaluated its impact on protein expression by using *L. lactis* VEL1153 (NZ9000 Δ*htrA*).

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *L. lactis* were grown in Difco™ M17 medium (Difco laboratory, USA) supplemented with 0.5 % glucose (GM17) at 30 °C under static conditions. *E. coli* DH5α strains were grown in Luria–bertani (LB) medium at 37 °C with vigorous shaking. Antibiotics were added at the indicated concentrations as necessary. Chloramphenicol and erythromycin were used at 10 and 2.5 μg/ml respectively for *L. lactis*. Chloramphenicol at 10 μg/ml and ampicillin at 100 μg/ml concentrations were used for *E. coli.*

DNA Manipulations

DNA manipulations were performed by using *E. coli* DH5α as an intermediate host by using standard procedures [6]. All Restriction Endonuclease (RE) enzymes (Thermo Scientific) were used as recommended by suppliers. PCR amplification using *Taq* DNA polymerase (Thermo Scientific) was performed.

Construction of pSEC:OmpA Vector

For construction of pSEC:OmpA, *ompA* sequence of *S. dysenteriae* type-1 was retrieved from NCBI (Gene accession number: 3799631) and was commercially synthesized from GenScript (GenScript, Hong Kong Ltd) in pUC57 cloning vector between *Nsi*I and *EcoR*V RE sites. pSEC:Nuc vector which was used as a backbone vector, has a combination of NICE system and usp45 secretory signal. As depicted in Fig. 1, *ompA* gene was cloned in

Table 1 Strains and plasmids used in the study

Strains and plasmids	Characteristics	References
Strains		
E. coli DH5α	F-φ80dlacZΔM15 Δ(lacZYA- argF)U169 endA1 recA1 hsdR17(rk− mk+) deoR thi-1 supE44 λ-gyrA96 relA1	Lab source
L. lactis MG1363	Wild type, Plasmid free	[24]
L. lactis NZ9000	MG1363 (*nisRK* genes into chromosome), plasmid free	[2]
L. lactis VEL1153 (NZ9000 Δ*htrA*)	NZ9000, functionally inactive HtrA (by Side Directed Mutagenesis)	[25]
L. lactis NZ9000 (pSEC:Nuc)	Cm^r, NZ9000 harboring pSEC:Nuc	This work
L. lactis NZ9000 (pSEC:OmpA)	Cm^r, NZ9000 harboring pSEC:OmpA	This work
L. lactis VEL1153 (pSEC:Nuc)	Cm^r, Ery^r, VEL1153 harboring pSEC:Nuc	This work
L. lactis VEL1153 (pSEC:OmpA)	Cm^r, Ery^r, VEL1153 harboring pSEC:OmpA	This work
Plasmids		
pUC57:OmpA	Amp^r, *ompA* gene was commercially synthesized in pUC57 plasmid	GenScript
pSEC:Nuc	Cm^r, usp-nuc fusion expressed under PnisA encoding the precursor usp45-Nuc (pre-Nuc)	[26]
pSEC:OmpA	Cm^r, derivative of pSEC:Nuc where *nuc* was replaced by *ompA* of *S. dysenteriae* type-1	This work

[a] Chloramphenicol (Cm^r) and erythromycin (Ery^r) were used at 10 and 2.5 μg/ml respectively for *L. lactis*. Chloramphenicol at 10 μg/ml and ampicillin (Amp^r) at 100 μg/ml concentrations were used for *E. coli*

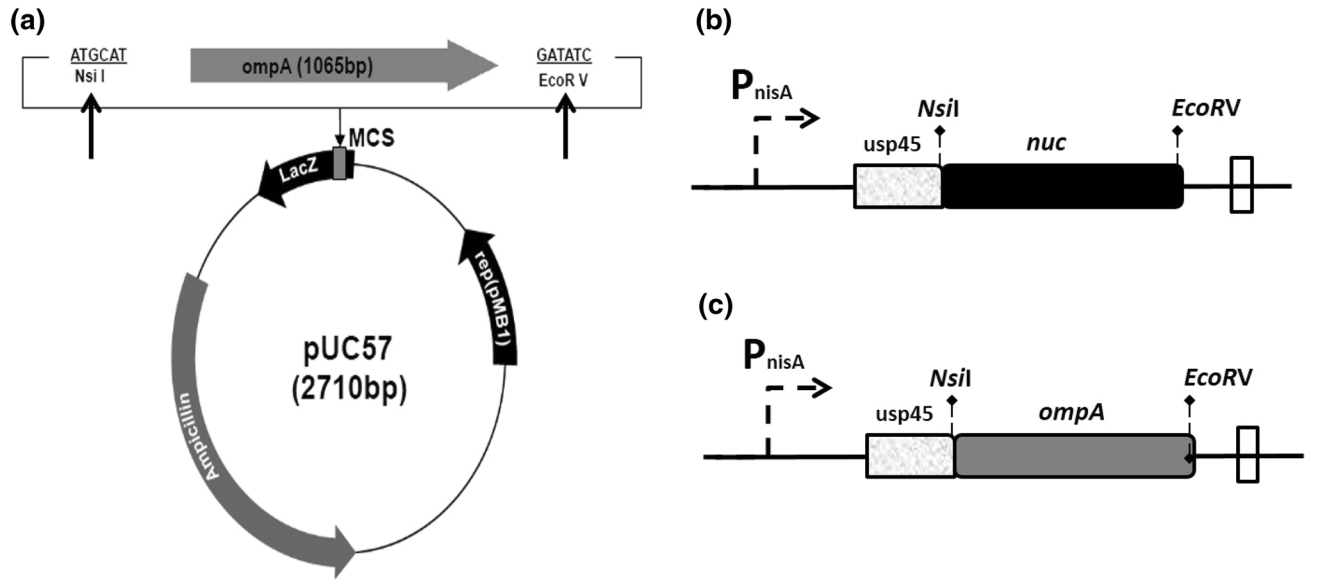

Fig. 1 Expression cassette for outer membrane protein A (OmpA) using the nisin inducible promoter (PnisA) and signal peptide usp45. **a** OmpA sequence of *S. dysenteriae* type-1 was commercially synthesized from GenScript in pUC57 cloning vector between *Nsi*I and *Eco*RV RE sites. **b** pSEC:Nuc vector which has a combination of NICE system and usp45 secretory signal was used as a backbone vector. **c** OmpA gene was cloned in place of nuc gene by using *Nsi*I and *Eco*RV RE sites and ligating the digested products. *Arrows* indicate presence of nisin inducible promoter PnisA. *Gray box* indicate presence of signal peptide of usp45 gene. *Small empty box* structure indicates trpA transcriptional terminator

place of *nuc* gene by using *Nsi*I and *Eco*RV RE sites. After ligating the digested products, it was used to transform *E. coli* DH5α. Transformants were screened by colony PCR using *ompA* specific primers (Forward 5′ GTTTCC TACCGTTTCGGTC 3′ and reverse 5′ TGCGCACTGAG AAGAAGAGA 3′) and verified by restriction sequence analyses.

Transformation of *L. lactis*

Lactococcus lactis NZ9000 and VEL1153 (NZ9000 Δ*htrA*) were transformed with pSEC:OmpA by electroporation. Transformed cells were plated onto GM17 agar containing chloramphenicol and incubated at 30 °C for 24 h. Transformants were screened by colony PCR using *ompA* and PnisA specific primers and preserved as glycerol stocks at −80 °C. Primers sequence for PnisA; Forward 5′ TGTC GATAACGCGAGCATAA 3′ and Reverse 5′ TCGAAAC AGATACCAAATCCAA 3′.

Expression of OmpA in *L. lactis*

For expression of OmpA, *L. lactis* strains harbouring the pSEC:OmpA were sub-cultured in fresh GM17 broth containing respective antibiotics and were grown statically at 30 °C till optical density at 600 nm was reached to 0.4–0.6. Cultures were then induced with different concentrations of nisin 2, 5, 10 and 15 ng/ml and were grown for 3 h. Halt Protease Inhibitor Cocktail (Thermo Scientific, IL USA) was added immediately after induction. Cells were harvested by centrifugation at 6000*g* for 10 min at 4 °C. Bacterial cell pellets as well as supernatant were processed as mentioned previously [3] with minor modifications. Briefly, cell pellet was resuspended in TES lysis buffer and incubated at 37 °C for 1 h. To break the cells, 50 µl of 20 % SDS was added and stored at −20 °C till further analysis. For precipitation of protein in supernatant two different precipitating agents; Tri-chloroaceticacid (TCA) and methanol were used. For precipitation by TCA method, 10 % TCA as a final concentration was added to the supernatant and incubated in ice for 30 min followed by centrifugation at 12,000*g* for 20 min at 4 °C. In case of methanol, supernatant was precipitated with three volumes of methanol for 2 h at 0 °C followed by centrifugation at 12,000*g* for 20 min at 4 °C. The protein pellet was recovered in 5X SDS gel loading dye and stored at −20 °C till further analysis.

RNA Isolation and Detection of OmpA Specific mRNA by Reverse Transcriptase PCR (RT-PCR)

RNA was isolated from *L. lactis* strains by RNA sure mini kit (Nucleo-pore, Genetix, India) using manufacturer's protocol. RT-PCR was performed using 1 µg of total RNA with Maxima cDNA synthesis kit (Thermo Scientific, USA). OmpA transcripts were detected with *ompA* specific

primers (Forward 5′ GTTTCCTACCGTTTCGGTC 3′ and reverse 5′ TGCGCACTGAGAAGAAGAGA 3′).

Western Blot Analysis

For western blot analysis, protein samples were separated by using 12 % SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore) by electro-blotting. The membrane was blocked for 1 h at room temperature in Phosphate buffer saline (PBS) containing 5 % skimmed milk. Membrane was then incubated with 1° Antibody raised against outer membrane proteins of *Shigella flexneri* (Outer membrane protein A of *S. flexneri* and *S. dysenteriae* type-1 are having 95.98 % similarity. For detailed description, refer Supplementary Fig. S5) (1:500 dilutions) for 1 h, followed by three 5 min washes in PBS. Peroxidase conjugated goat anti-rabbit (Calbiochem, USA) was added at 1:1000 dilutions to the membrane and incubated for 1 h at room temperature followed by three 5 min PBS washes. For detection of antigen, TMB substrate was used (Invitrogen, Carlsbad, USA) and blot was visualized by using Gel Doc^{TM} XR^{+} System (Bio Rad Laboratory, USA).

Results

Construction, Transformation and Expression

To facilitate the controlled secretion of antigenic protein OmpA by *L. lactis*, we have constructed *E. coli–L. lactis* shuttle vector pSEC:OmpA. Construction of pSEC:OmpA is schematically represented in Fig. 1. *ompA* gene of *S. dysenteriae* type-1 is flanked by *Nsi*I and *EcoR*V in pUC57:OmpA. Both the vectors pUC57:OmpA and pSEC:Nuc were double digested with *Nsi*I and *EcoR*V resulting in the restriction fragments of 1071 and 3275 bp corresponding to *ompA* gene and pSEC backbone respectively as depicted in Fig. S1. Ligation of digested products were then used to transformed *E. coli* DH5α. Obtained clones were screened by colony PCR showing amplicon of 252 bp corresponding to presence of *ompA* (data not shown). Clones were verified by restriction sequence analysis as depicted in Fig. S2. Transformation in *L. lactis* strains NZ9000 and VEL1153 (NZ9000 Δ*htrA*) was done by electroporation and confirmed by colony PCR using *ompA* and P*nisA* specific primers as shown in Fig. S3(a) and S3(b). Further verification of pSEC: OmpA was done by restriction sequence analysis as shown in Fig. S3(c). *ompA* transcripts were confirmed by the presence of 151 bp amplicon as shown in Fig. S4. Evaluation of OmpA expression was then carried out by western blot.

OmpA Expression

Expression of heterologous protein was described by Ribeiro et al., [7], we followed the same for OmpA expression with minor modification in nisin concentration i.e. 10 ng/ml. In order to determine whether the OmpA protein secreted out or remain inside the cell, protein was precipitated from the cell pellet and supernatant that were further analyzed by Western blot. When proteins were precipitated with TCA method, as anticipated, band of OmpA was not detected in cell pellet (data not shown). To our surprise, it was also absent in supernatant as shown in Fig. 2. Here, OmpA protein of *S. dysenteriae* type-1 purified from r-*E. coli* BL21 (DE3)::pET28-OmpA was used as positive control (Supplementary Fig. S6). To resolve the ambiguity of OmpA expression, optimization of the factors involved in its expression and detection were assessed.

Optimization of Factors

Protein Precipitating Agent

Precipitation of protein by TCA method has been very well documented in case of *L. lactis* expression system [1, 8]. However, we failed to see any band in cell pellet and even in supernatant when TCA was employed for precipitation; hence TCA was replaced with methanol. As depicted in Fig. 2, ~74 kDa protein band which is twice the molecular weight of desired protein was observed along with the degraded protein adducts when precipitated with methanol.

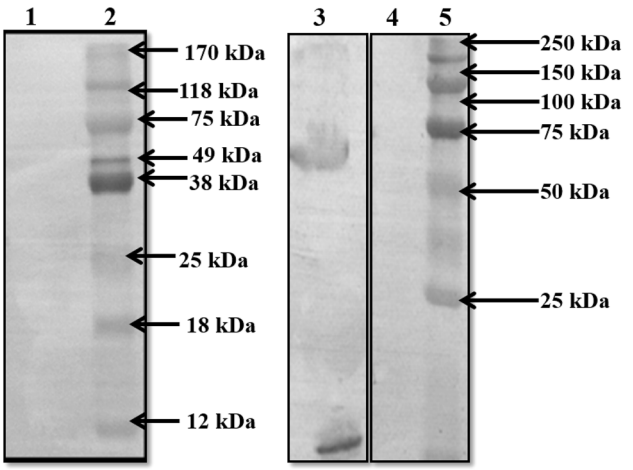

Fig. 2 Detection of expressed OmpA by comparing methanol and TCA as precipitating agent upon induction at 10 ng/mL. *Lane-1* Cell pellet of induced *r*-NZ9000 (pSEC:OmpA) precipitated with methanol, *Lane-2* ProSieve color protein marker, Lonza Rockland, *Lane-3* Supernatant of induced *r*-NZ9000 (pSEC:OmpA) precipitated with methanol, *Lane-4* Supernatant of induced *r*-NZ9000 (pSEC:OmpA) precipitated with TCA, *Lane-5* Precision plus protein standard, BIORAD

Inducer (Nisin)

Inducer (nisin) activates *nisRK* regulator, a two component system, followed by activation of promoter PnisA cascade. For controlled expression of several heterologous proteins, nisin concentration as low as 0.025 ng/ml [2] and as high as 500 ng/ml has been reported [9]. Hence, it provides a wide arena for optimisation of nisin concentration in accordance to the expressed protein.

To start the optimisation 2, 5, 10 and 15 ng/ml nisin concentrations were tested initially to attain the optimum expression of OmpA. As described in Fig. 3, lane 3 illustrates aggregated protein product of ~74 kDa, at 2 ng/ml nisin concentration. In Lane 4, the aggregated ~74 kDa protein band and degraded protein products ranging from ~15 kDa were observed after induction at 10 ng/ml nisin concentration. Lane 1 exhibits the lower molecular weight protein bands indicating the degradation caused by induction at higher concentration of nisin i.e. 15 ng/ml. With increase in inducer concentration, there is concomitant increase in degradation of protein product.

Proteases in Protein Expression

Proteolytic degradation is one of the limiting factor for stable production of heterologous protein by NICE system [10]. Till date, ClpP (intracellular) and HtrA (extracellular) have been identified as major proteases in *L. lactis* strains [11]. To prevent protein degradation, we incorporated protease inhibitor cocktail during the expression of OmpA, as a foremost measure to prevent protein degradation.

As shown in Fig. 4a, the expressed protein of desired size, devoid of any degraded protein products was observed. Indeed, the presence of ~35 kDa band just below the desired protein band of 37 kDa, demonstrates the inefficient prevention against proteases.

HtrA is a trypsin like serine protease which degrades misfolded protein at cell surface [11]. Therefore, the existing strategy was improvised by expressing OmpA in HtrA deficient strain to combat the inevitable degradation. As shown, in Fig. 4b, sharp band of desired size 37 kDa was obtained with *r*-VEL1153 (NZ9000 Δ*htrA*). This result indicates the role of HtrA in degradation of OmpA along with other intracellular proteases. Optimum expression of heterologous protein in *L. lactis* thus depends on critical factors as mentioned previously.

Discussion

Accomplishing successful construction of pSEC:OmpA, expression of OmpA in *L. lactis* has been explored using NICE. Although it is widely used, knowledge about variable factors and their integrative effects in modulating expression of heterologous protein is scarce. Despite the presence of OmpA specific transcripts, which provided the experimental evidence for the transcription of OmpA, challenge remained at the level of detection of the expressed protein. As protein expression at post-transcriptional level is affected by several factors [12], we have evaluated the crucial factors viz. protein precipitating agents, inducer concentration and presence or absence of host proteases in expression of OmpA.

When we attempted precipitation of OmpA protein with TCA method, we failed to see any band in Western blot. This might be due to the degradation of expressed protein [8], loss of protein during acetone wash and/or incomplete solubilization of precipitated proteins [13, 14]. However,

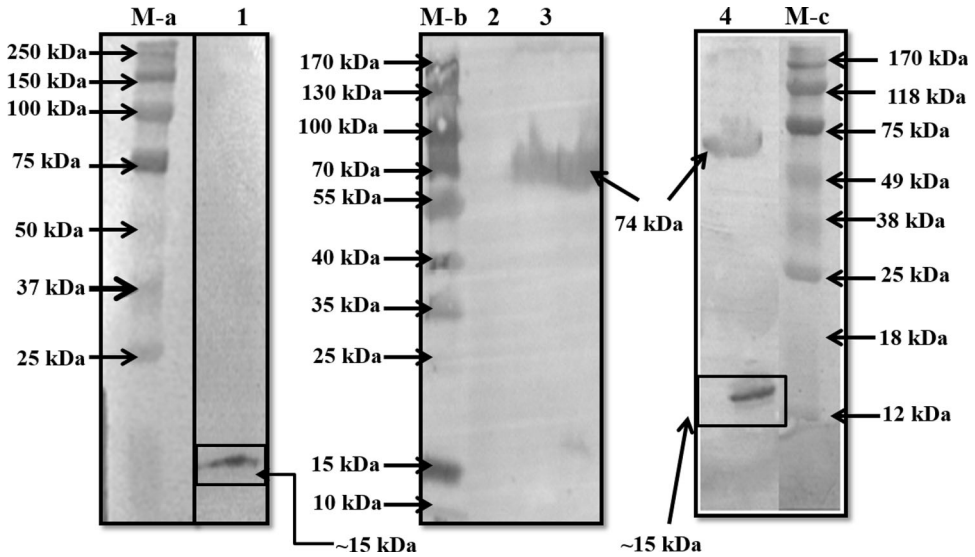

Fig. 3 Expression of OmpA at different nisin (inducer) concentration. *Lane M-a* Precision plus protein standard, BIORAD, *Lane-1 r*-NZ9000 (pSEC:OmpA) induced with 15 ng/mL, *Lane-M-b* Pre-stained protein ladder, Thermo Scientific, *Lane-2* Empty, *Lane-3 r*-NZ9000 (pSEC:OmpA) induced with 2 ng/mL, *Lane-4 r*-NZ9000 (pSEC:OmpA) induced with 10 ng/mL, *Lane-M-c* ProSieve Color Protein Marker, Lonza Rockland

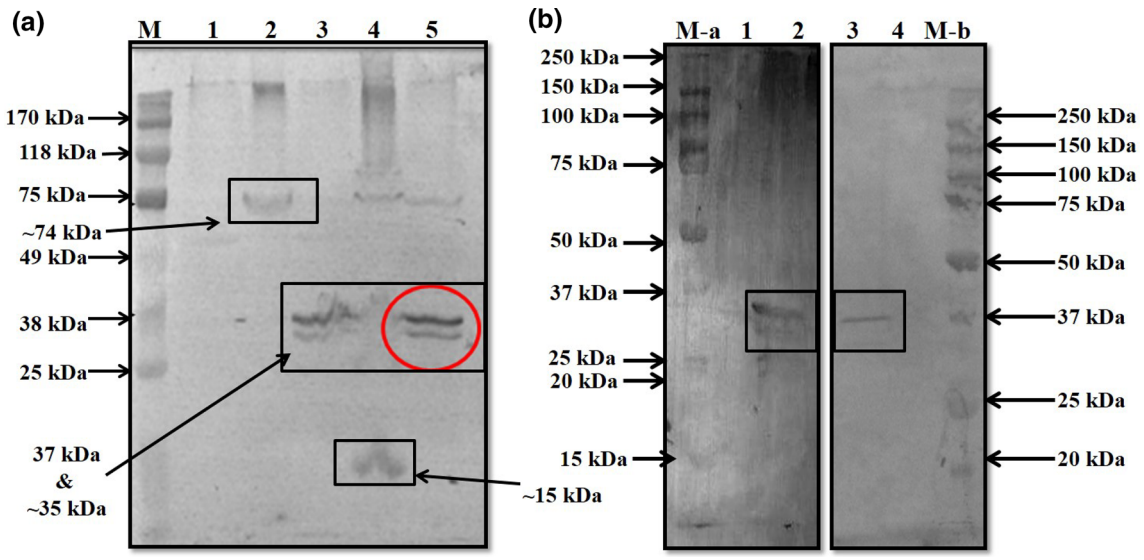

Fig. 4 Effect of nisin concentrations, protease inhibitor cocktail and Δ*htrA* strain on protein expression. **a** *M* ProSieve color protein marker, Lonza Rockland, *Lane 1* Uninduced *r*-NZ9000 (pSEC:OmpA), *Lane 2–5 r*-NZ9000 (pSEC:OmpA) induced respectively with 2, 5, 10 and 15 ng/ml nisin and protease cocktail. **b** *Lane M-a*, *M-b* Precision plus protein standard, BIORAD, *Lane-1* Empty, *Lane-2 r*-VEL1153 (pSEC:OmpA) induced with 5 ng/mL, *Lane-3 r*-VEL1153 (pSEC:OmpA) induced at 5 ng/mL with protease cocktail, *Lane-4* Uninduced *r*-VEL1153 (pSEC:OmpA)

when we switched from TCA to methanol, we observed band of ~74 kDa which is twice the size of OmpA (37 kDa). Though, under denaturing condition, it is unlikely that protein exist in dimer or aggregated form. However, there are certain membrane proteins such as KcsA, β-Glycosidases, and KvLm *K*+ channels which remains in the dimer or aggregated form [15, 16]. These reports strengthen the observation obtained with OmpA. Along with the aggregation, degraded protein product was also found which may be the consequence of the presence of proteases in host strain NZ9000 [11, 17] and/or degradation of highly expressed protein [18].

In 2006, Zhou et al. [17] reported the linear dose dependent relationship suggesting elevated protein expression at higher nisin concentration. Further, overexpression of membrane proteins also induces stress response in *L. lactis*, resulting in protein degradation by chaperons and proteases [19]. Induction at 5, 10 and 15 ng/ml nisin concentrations revealed the degraded pattern of expressed proteins eliciting the possibility of degradation at higher nisin concentration resulting in increased expression and susceptibility to proteases.

Due to the presence of surface as well as intracellular host proteases, considerable degradation of highly expressed proteins has been observed [20]. Such reports lead us to explore the role of proteases in heterologous protein expression.

Existence of house-keeping proteases in *L. lactis* makes the heterologous proteins susceptible to degradation. Due to the consistent protein degradation observed in our results, we explored protease inhibitors as a remedial tool. Extensive usage of protease inhibitor cocktail has been demonstrated to combat protein degradation [21] which upon incorporation in our study, prevented the degradation from broad range of proteases. However, presence of 35 kDa band reflected the need to identify the candidate responsible for degradation of our protein.

Expression of ample number of proteins viz. *Staphylococcus aureus* nuclease, *Staphylococcus hyicus* lipase, bovine rotavirus non-structural protein 4 NSP4 [20], human papillomavirus antigen E7 [22] and Brucella abortus antigen L7/L12 [7] were found to be degraded in host strain NZ9000. Protein degradation in aforesaid cases is eliminated by carrying out expression in HtrA deficient strains. When expression of OmpA in *r*-VEL1153 (NZ9000 Δ*htrA*) strain was carried out, we achieved 37 kDa band devoid of any degraded protein product. The obtained results strengthen the role of HtrA as a key extracellular protease.

In addition to HtrA, proteases like ClpP and several other housekeeping proteases which are poorly understood [23], were inhibited by incorporation of protease inhibitor cocktail. Such findings in our study suggest the critical role of proteases in efficient protein expression.

Altogether, the present study revealed the role of protein precipitating agent, inducer concentration and intracellular as well as extracellular proteases in heterologous protein expression by *L. lactis*. Our result demonstrates the combination of *htr*A mutant strains, protease inhibitor

cocktails, lower nisin inducer concentration and methanol as protein precipitating agent giving optimum expression of heterologous membrane protein. This report outlines key factors and their integrative effects in modulating expression of heterologous protein using NICE.

In nutshell, present work will contribute to the better understanding of factors affecting heterologous protein expression using NICE in *L. lactis*.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest with respect to authorship, funding and publication of this article.

References

1. Cortes-Perez NG, Poquet I, Oliveira M, Gratadoux JJ, Madsen SM, Miyoshi A, Corthier G, Azevedo V, Langella P, Bermúdez-Humarán LG (2006) Construction and characterization of a *Lactococcus lactis* strain deficient in intracellular ClpP and extracellular HtrA proteases. Microbiology 152:2611–2618. doi:10.1099/mic.0.28698-0
2. Kuipers OP, De Ruyter PGGA, Kleerebezem M, De Vos WM (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotechnol 64:15–21. doi:10.1016/S0168-1656(98)00100-X
3. Villatoro-Hernandez J, Loera-Arias MJ, Gamez-Escobedo A, Franco-Molina M, Gomez-Gutierrez J, Rodriguez-Rocha H, Gutierrez-Puente Y, Saucedo-Cardenas O, Valdes-Flores J, Montes-de-Oca-Luna R (2008) Secretion of biologically active interferon-gamma inducible protein-10 (IP-10) by *Lactococcus lactis*. Microb Cell Fact 7:22. doi:10.1186/1475-2859-7-22
4. Le Loir Y, Azevedo V, Oliveira SC, Freitas DA, Miyoshi A, Bermúdez-humarán 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. Microb Cell Fact 13:1–13. doi:10.1186/1475-2859-4-2
5. Frelet-barrand A, Boutigny S, Kunji ERS, Rolland N (2010) Heterologous expression of membrane proteins. Methods Mol Biol 601:67–85. doi:10.1007/978-1-60761-344-2
6. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
7. Ribeiro LA, Azevedo V, Le LY, Oliveira SC, Dieye Y, Piard JC, Gruss A, Langella P (2002) Production and Targeting of the Brucella abortus Antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. Appl Environ Microbiol 68:910–916. doi:10.1128/AEM.68.2.910
8. Zellner M, Winkler W, Hayden H, Diestinger M, Eliasen M, Gesslbauer B, Miller I, Chang M, Kungl A, Roth E, Oehler R (2005) Quantitative validation of different protein precipitation methods in proteome analysis of blood platelets. Electrophoresis 26:2481–2489. doi:10.1002/elps.200410262
9. Oddone GM, Mills DA, Block DE (2009) Incorporation of nisI-mediated nisin immunity improves vector-based nisin-controlled gene expression in lactic acid bacteria. Plasmid 61:151–158. doi:10.1016/j.plasmid.2008.12.001
10. Rigoulay C, Poquet I, Madsen SM, Gruss A (2004) Expression of the Staphylococcus aureus surface proteins HtrA1 and HtrA2 in *Lactococcus lactis*. FEMS Microbiol Lett 237:279–288. doi:10.1016/j.femsle.2004.06.046
11. Oddone GM, Mills DA, Block DE (2009) Dual inducible expression of recombinant GFP and targeted antisense RNA in *Lactococcus lactis*. Plasmid 62:108–118. doi:10.1016/j.plasmid.2009.06.002
12. Desai PN, Shrivastava N, Padh H (2010) Production of heterologous proteins in plants: strategies for optimal expression. Biotechnol Adv 28:427–435. doi:10.1016/j.biotechadv.2010.01.005
13. Jiang L, He L, Fountoulakis M (2004) Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. J Chromatogr A 1023:317–320. doi:10.1016/j.chroma.2003.10.029
14. Fic E, Kedracka-Krok S, Jankowska U, Pirog A, Dziedzicka-Wasylewska M (2010) Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. Electrophoresis 31:3573–3579. doi:10.1002/elps.201000197
15. Bowie JU (2005) Solving the membrane protein folding problem. Nature 438:581–589. doi:10.1038/nature04395
16. Gentile F, Amodeo P, Febbraio F et al (2002) SDS-resistant active and thermostable dimers are obtained from the dissociation of homotetrameric β-glycosidase from hyperthermophilic Sulfolobus solfataricus in SDS: stabilizing role of the A–C intermonomeric interface. J Biol Chem 277:44050–44060. doi:10.1074/jbc.M206761200
17. Zhou XX, Li WF, Ma GX, Pan YJ (2006) The nisin-controlled gene expression system: construction, application and improvements. Biotechnol Adv 24:285–295. doi:10.1016/j.biotechadv.2005.11.001
18. Drouault S, Corthier G, Ehrlich SD (2000) Expression of the Staphylococcus hyicus Lipase in *Lactococcus lactis*. Appl Environ Microbiol 66:588–598. doi:10.1128/AEM.66.2.588-598.2000
19. Marreddy RKR, Pinto JPC, Wolters JC, Geertsma ER, Fusetti F, Permentier HP, Kuipers OP, Kok J, Poolman B (2011) The response of *Lactococcus lactis* to membrane protein production. PLoS ONE 6:1–15. doi:10.1371/journal.pone.0024060
20. Enouf V, Langella P, Commissaire J, Cohen J (2001) Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. Appl Environ Microbiol 67:1423–1428. doi:10.1128/AEM.67.4.1423
21. Bodzon-Kulakowska A, Bierczynska-Krzysik A, Dylag T, Drabik A, Suder P, Noga M, Jarzebinska J, Silberring J (2007) Methods for samples preparation in proteomic research. J Chromatogr, B: Anal Technol Biomed Life Sci 849:1–31. doi:10.1016/j.jchromb.2006.10.040
22. Langella P, Miyoshi A, Gruss A, Guerra RT, Oca-luna RMDe, Le LY (2002) Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. Appl Environ Microbiol 68–2:917–922. doi:10.1128/AEM.68.2.917
23. Frees D, Ingmer H (1999) ClpP participates in the degradation of misfolded protein in *Lactococcus lactis*. Mol Microbiol 31:79–87
24. Gasson MJ (1983) Genetic transfer systems in lactic acid bacteria. Antonie Van Leeuwenhoek 49:275–282. doi:10.1007/BF00399500
25. Foucaud-Scheunemann C, Poquet I (2003) HtrA is a key factor in the response to specific stress conditions in *Lactococcus lactis*.

FEMS Microbiol Lett 224:53–59. doi:10.1016/S0378-1097(03)00419-1

26. Bermdez-HumarÃ¡n LG, Langella P, Commissaire J, Gilbert S, Loir Y, L'Haridon R, Corthier G (2003) Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in Lactococcus lactis. FEMS Microbiol Lett 224:307–313. doi:10.1016/S0378-1097(03)00475-0