BIOREACTOR STRATEGIES TO INCREASE THE ENGINEERED PROTEIN PRODUCTION IN LACTOCOCCUS LACTIS

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Abstract

Lactococcus lactis is a lactic acid bacterium widely used in various food and fermentation processes. *L. lactis* is one of the organism which is treated as generally regarded as safe (GRAS) organisms, whichch is used for as genetic machinery for the genetic and chromosomal engineering and vectors to facilitate cloning and gene expression and produces a number of important proteins. However, the production of large quantities of biologically active recombinant proteins in this organism is restricted due to a number of factorsand biological constraints. One of the constraints with this system is that, in glucose growing culture, *L. lactis* develops a stress response and cessation of growth due to the acidification of medium. Prior work in our laboratory has shown that suppression of this response increases the streptokinase productivity in *L.lactis*. It was shown that buffering the medium suppresses development. In this work, the correlation between ATR suppression and increase in recombinant protein activity was used to develop bioreactor strategies for enhancement of volumetric activity of recombinant streptokinase.

Keywords : Lactic acid, lactobacillus, fermentation, recombinant, streptokinase.

Introduction

Advancements in genetic engineering and bioprocess technology have seen the development of several potent expression systems for production of recombinant proteins. These recombinant proteins have wide applications in therapy, diagnostics and industrial processes (Kar *et al*., 2016; Farooq and Sehgal, 2019a, 2019b). These expression systems include Gram-positive and Gram negative prokaryotes, yeasts, filamentous fungi, plant cell, insect cell and mammalian cell cultures as well as in-vitro expression. All the expression systems employed so far have certain advantages as well as disadvantages that should be considered during their selection (Rai *et al*., 2001; Singh and Das, 2017). An effective process for the production of recombinant proteins is characterized by increased product as well as biomass (Riesenberg and Guthke, 1999; Chouhan, *et al*., 2017; Malkania *et al*., 2018). Among the several expression systems available, bacterial expression systems are more amenable to the condition of high specific-productivity, concomitant with high cell concentrations in fermentation processes. Therefore, they have been widely used on the industrial scale for the production high value recombinant proteins (Singh *et al*., 2016; Singh and Thakur, 2018; Singh *et al*., 2019).

The design of prokaryotic expression vectors has been extensively investigated (Makrides, 1996) and various essential features of the expression vector has been identified and optimized for better performance (Malik *et al*., 2013; Kaur *et al*., 2016; Malik *et al*., 2016). The selection of the expression systems also depends on the properties of the recombinant protein to be over-expressed. Even though optimized expression strains and vectors are available for all expression systems developed there is a lack of universally-employed host-vector system for high-level expression of heterologous proteins (Kumar *et al*., 2019; Kaur *et al*., 2020; Prabhakar *et al*., 2020).

A lactic acid bacterium, Lactococcus lactis is used very frequently in the food processing industries for making cheese, buttermilk, as preservative, for probiotics etc (Mishra *et al*., 2018; Mishra, 2019a, 2019b). *L. lactis* is one of the organism which is treated as generally regarded as safe (GRAS) organisms, whichch is used for as genetic machinery for the genetic and chromosomal engineering and vectors to facilitate cloning and gene expression and produces a number of important proteins (Sharma *et al*., 2017). However, the production of large quantities of biologically active recombinant proteins in this organism is restricted due to a number of factorsand biological constraints (Le Loir *et al*., 2005). Recombinant proteins can be synthesized and targeted to different compartments in *L. lactis* (Table-1) (Nauta *et al*., 1996; O'Sullivan *et al*. 1996; Sanders *et al*., 1997, 1998; Kuipers *et al*., 1998).

Table 1 : Recombinant proteins produced in *Lactococcus lactis*. (S: secreted, C: cytoplasmic, A: Anchored).

Proteins	Gene	Origin	Localization	References
Bacterial antigens				
L7/L12	*L7/L12*	*Brucella abortus*	S/C/A	Ribeiro *et. al.,* 2002
TTFC	*ttfc*	*Clostridium tetani*	S	Wells *et al.,* 1993
Viral antigens				
E7	*E7*	HPV type 16	S	Bermudez-Humaran *et. al.,* 2002
NSP4	*NSP4*	*Bovine rotavirus*	C	Enouf *et. al.,* 2001
BCV epitope	*BCV*	*Bovine coronavirus*	S	Langella and Le Loir, 1999
LL-CRR	*LL-CRR*	*Streptococcus pyogenes*	S	Mannam et. al., 2004
Cytokines				
IL-2	*IL-2*	mice	S	Steidler *et. al.,* 1995
IL-6	*IL-6*	mice	S	Steidler *et. al.,* 1998
IL-10	*IL_10*	mice	S	Schotte *et. al.,* 2000
IL-12	*IL-12*	mice	S	Bermudez *et. al.,* 2002
Enzymes				
Lipase	*lip*	*Staphylococcus hycius*	S	Drouault et. al., 2002
Streptokinase	*stk*		S	Sriraman and Jayaraman, 2006

Compared to conventionally used expression system *E. coli, L. lactis* has several advantages. *L. lactis* does not produce endotoxins, which makes it a potentially safe expression system when recombinant proteins are used as therapeutics and other health related applications. Secretion of recombinant proteins is much easier in *L. lactis* compare to *E.coli*. During stressful environmental conditions the proteases and chaperones secreted by *L. lactis* is less in number compared to *E.coli*, which reduces the degradation of recombinant proteins (Laskowska *et al.*, 1996). Many heterologous eukaryotic, prokaryotic and viral proteins have been produced and secreted by *L. lactis*. Table 1 describes different recombinant proteins expressed in *L. lactis*.

L. lactis based Expression systems

Several expression systems based on constitutive and inducible promoter have been developed in *L. lactis* for production of recombinant proteins (Morello *et al.*, 2008; Nankar *et al.*, 2017; Prasher *et al.*, 2018). Inducible promoters are widely used for large scale production of recombinant proteins. These inducible promoter systems have several advantages which includes high efficiency of the promoter which allow high level expression, tight regulation which reduces the possibility of growth inhibition, easily adaptable to large scale production processes and secretary expression which allows for ease of protein purification (Prabhakar *et al.*, 2013; Prabhakar *et al.*, 2014; Prabhakar *et al.*, 2020). Several other inducible promoter systems which are used in *L. lactis* include temperature inducible systems, aeration inducible systems, acid inducible systems, nisin inducible systems etc (Djordjevic *et al.*, 1998; Mierau *et al.*, 2005).

(a) *P170 Promoter*

"P170 is a derivative of a native *L. lactis* promoter, identified during screening for environmentally regulated promoters (Israelsen *et al.*, 1995). The promoter is regulated by pH and growth phase i.e. the activity is strongly up-regulated at pH below 6.5 during the transition to stationary phase, without the need for addition of an exogenous inducer (Madsen *et al.*, 1999). Consequently, the growth phase is separated from the protein production phase. P170 promoter consist of a novel 14 bp regulatory DNA region centred at around the -41.5 region and composed of three tetra

nucleotide sequences, boxes A, C and D (ACiD Box). The promoter has an extended -10 region and lacks the typical -35 region (Paul *et al.*, 2018)."

"Deletion analysis of P170 promoter revealed a minimal promoter region of 51bp required for both promoter activity and pH regulation (Madsen *et al.*, 1999). P170 segment contains a cis-acting sequence involved in the control of promoter regulation (Sharma *et al.*, 2019). Transcriptional analysis showed that P170 is responsible for the transcription of a monocistronic gene orfX which encodes a polypeptide homologous to a hypothetical protein from *Bacillus subtilis*. Deletion analysis and chemical mutagenesis of P170 defined a specific region within the un-translated mRNA leader that is able to modulate the expression level directed by the P170 promoter."

"Deletion of a 72 bp HaeIII fragment from this leader region resulted in a 150-fold to 200-fold increase in the level of gene expression, without affecting the regulation. RcfB, a trans activating protein, is involved in P170 basal activity and is essential for pH induction. P170 is a strong promoter, only active at low pH and when cells enter the stationary growth phase. It has the property of being more active at low than at high temperatures (Israelsen *et al.*, 1995; Madsen *et al.*, 1999).There are few examples in literature on recombinant protein production using the P170 expression system. Glenting *et al.* (2007) have cloned a synthetic ara h 2 gene into an *L. lactis* expression plasmid containing the P170 promoter and the SP310mut2 signal sequence. Fermentation using CHW9 and synthetic medium gave a maximum cell density of 12 OD_{600}. As the cell density increased and reached transition to stationary phase the P170 promoter was induced and a ~17 kDa secreted product was accumulated in the culture supernatant. A batch fermentation resulted in 40 mg/L recombinant Ara h 2 (Glenting *et al.*, 2007)."

(b) Nisin-inducible expression system

Nisin-inducible expression system is widely used for production of recombinant proteins in *L. lactis* (Mierau and Kleerebezem 2005). The promoter is activated in response to an anti-microbial peptide called nisin. Nisin is encoded by a cluster of 11 genes, of which the first gene, nisA, encodes the precursor of nisin. The other genes (nisB, nisC, nisI, nisP, nisE, nisF, nisG) help in the modification of nisin precursor,

translocation and processing of nisin, immunity against nisin and in the regulation of nisin synthesis. The synthesis of nisin is regulated by two proteins known as NisK and NisR. NisK senses the presence of nisin in the medium and auto-phosphorylates and interacts with NisR which functions as transcriptional activator and regulates the transcription of PnisA and PnisF promoters and thereby the synthesis of nisin. These NisK and NisR proteins are constitutively expressed and belong to a family of bacterial two-component regulatory signal transduction systems (Kuipers *et al.*, 1998).

Nisin inducible strain of *L. lactis* has been developed, where nisK and nisR genes were inserted into the chromosome of *L. lactis* and NZ9000 strain was created (Kuipers *et al.,* 1998). The genes of interest are placed under the PnisA promoter in the plasmid. The induction of promoter is determined by the sub-inhibitory addition of nisin in the culture medium and the induction strength is directly proportional to the concentration of nisin used in the medium. Nisin controlled gene expression was used to develop a recombinant strain of *L. lactis* that is able to express the Pneumococcal protective protein A (PppA) on its surface. *L. lactis* was engineered to express Giardia lamblia cyst wall protein 2 (CWP2) at three different sub-cellular locations - intracellular, secreted or cell-surface-anchored, using nisin as an inducing agent (Lee and Faubert, 2006; Mann *et al.*, 2019).

A subunit of VP4 capsid protein called VP8 was expressed and secreted to cell surface of *L. lactis*. For this purpose, a secretion vector has been constructed with the Lactococcal signal sequence AL9 and the VP8 encoding gene fragment (Bedi *et al.*, 2019).

The amount of VP8 secreted by *L. lactis* in the culture supernatant was quantified and visualized by Western blot. The amount of VP8 protein in 30-fold concentrated supernatant was 0.1- 0.2 mg.ml^{-1}.

From the examples given above, it can be seen that recombinant protein production in *L. lactis* strains can be several hundred to several thousand fold lower in comparison to *E. coli*. Despite the knowledge and techniques, over-production of recombinant proteins in *L. lactis* is often subject to various biological and process constraints which have often lead to different levels of success. These constraints were observed to be a strong function of the recombinant protein, localization within the cell and its cellular transport.

Genetic strategies to improve cellular productivity

Various modifications of the host system and expression vectors have been carried out to improve the production of recombinant protein in *Lactococcus lactis*. The most critical factor considered for increasing the production is the localization of protein produced. The overall improvement of the productivity depends on the kind of modifications at genetic level and the physico-chemical properties of the recombinant protein produced. Due to the overproduction of recombinant protein sometimes cells undergo metabolic stress. During stressful conditions cells synthesize several proteases which degrade the recombinant protein. Over expression of plasmid encoded genes triggers transcription of heat-shock genes and other stress responses and often results in the aggregation of encoded proteins as inclusion bodies. The formation of these deposits represents a major obstacle for the production of recombinant proteins and restricts the proteins being available for industrial purpose (Carrio *et al.*, 2005). The over accumulation of recombinant proteins is sometimes toxic to the cells and affects the normal metabolic activity of cells and causes cell death (Tomoko and Murata, 1995). Several intracellular and surface proteases are present in *L. lactis* which degrades the recombinant proteins under stressful conditions.

Different recombinant proteins have been produced in *L. lactis*, where the proteins have been localized at different cellular locations (Le Loir *et al.*, 2005). They have increased production yield of secretary recombinant proteins by genetic modification of signal peptides and thereby reducing the degradation by intracellular proteases. This has resulted in better activity as compared to intracellular localization.

Though secretary expression systems increase the yield of proteins, specific applications need different cellular localizations. In industry secretary expression systems are widely used to increase the yield. Norton *et al.* (1996) used *L. lactis* to express fragment C of tetanus toxin and localized the protein to cell surface. They found that the surface anchored tetanus toxin fragment C increased the immunogenicity by 10-20 fold compare to intracellular or secretory expression systems of *L. lactis*.

Secretion of proteins has been improved by co-expressing chaperone like proteins in *L. lactis*. Co expression of pmpA, a PrsA like protein has shown to increase the stability of secreted *S. hycicus* (Drouault *et al.*, 2002). Lindholm *et al* (2006) have shown similar kind of result where co-expression of *Bacillus subtilis* PrsA, a chaperone-like protein improved the yield of amylase by 20-fold in *L. lactis*. *L. lactis* has an unique surface housekeeping protease belong to HtrA/DegP family. It has been found to be a key factor during various stresses like high temperature, ethanol etc (Poquet et. al., 2000, Foucoud-Scheunemann and Poquet 2003). HtrA is a trypsin-like serine protease essential for growth at high temperatures (39 °C for *L. lactis*) and which degrades misfolded proteins at the cell surface (Foucaud-Scheunemann & Poquet, 2003). Miyoshi *et al*. (2002) have shown increased stability of recombinant proteins in *htrA-L. lactis*. In this study they expressed *Staphylococcus hyicus* lipase, the bovine rotavirus antigen non-structural protein 4, human papillomavirus antigen E7 and *rucella abortus* antigen L7/L12 in *htrA-L. lactis*. In all cases degradation of proteins was significantly lower although the over all production was lower. Sriraman (2008) has found that production of recombinant streptokinase in *htrA-L. lactis* prevented proteolysis of streptokinase (found in the wild-type in *L. lactis* MG1363). However, secretion efficiency was found to be poor with the *htrA-L. lactis* strain (Saxena *et al.*, 2016).

A major disadvantage associated with HtrA mutant is its growth rate is affected at high temperatures (Foucoud-Scheunemann and Poquet, 2003). *L. lactis* has an unique cytoplasm housekeeping protease known as ClpP which causes intracellular degradation of abnormally expressed proteins during various stressful conditions like high temperature, lethal acidic pH etc (Frees and Ingmer 1999). Cortes-Perez *et al*. (2006) have used *htrA-clpP* double mutant strain of *L. lactis* for production of staphylococcal nuclease (Nuc) and the human papillomavirus E7 protein. Comparison between the *clpP-htrA* double mutant and the

wild-type control strain revealed reduced proteolytic activities and increased stability of these two recombinant proteins.

There are several other ways to improve the cellular productivity of recombinant protein. The productivity can also be increased by optimizing medium compositions, modifying environmental parameters and by appropriate bioreactor strategies. Sriraman and Jayaraman (2006) have developed a novel strategy to improve recombinant protein productivity in *L. lactis*, by suppressing the onset of acid tolerance response (ATR) which develops during acidification of the medium. They found that development of ATR, due to increased acidification of the medium in glucose-grown cultures, leads to up-regulation of the surface protease HtrA and increased degradation of streptokinase which reduces productivity. Suppression of ATR, by appropriate buffering of the medium, led to a several fold increase in recombinant protein production as well as reduced degradation due to down-regulation of *htrA* expression. Since ATR suppression will be one of the key variables addressed in this work for improving recombinant protein productivity in bioreactors, it is important to understand the molecular and biochemical basis of this phenomenon in *L. lactis* as well as the significance of this stress response on cellular growth and metabolism.

Acid tolerance response

Microorganisms can adapt to different environmental stresses. One of the frequent stress conditions encountered by microorganism is acid pH. In response to low environmental pH the bacteria develop an acid inducible stress response called acid tolerance response (ATR). ATR is especially important for bacteria, such as lactic acid bacteria (LAB) that produce organic acids as metabolic end products and acidify their own environment. Development of ATR has been reported for different strains of *L. lactis* (Hartke *et al*., 1996, O'Sullivan and Condon 1999, Rallu *et al*., 2000). In *L. lactis* development of acid tolerance response induces synthesis of stress proteins, including a few heat-shock and UV-inducible proteins (Hartke et. al., 1996). For *L. lactis* ssp. *cremoris* strains (MG1363 and 712), acid adaptation needs *de novo* protein synthesis (Rallu *et al*., 1996) and results in increased survival from heat, ethanol, H_2O_2 and NaCl stress (O'Sullivan and Condon, 1999).

These observations suggest that acid stress adaptation overlaps with other stress responses and results in cross-protection. Regulation of the cytoplasmic pH is a fundamental requirement to withstand acidic environments. The cytoplasmic pH homeostasis is primarily maintained by means of a proton-translocating ATPase. Conditions that lead to acidification of the cytoplasm increase both the amount and activity of ATPase (O'Sullivan *et al*., 1999). It was shown that during the development of ATR the expression level of H^+ATPase is increased and helps in pH homeostasis to maintain the internal pH (pHi) which is a critical factor affecting ATR (O'Sullivan and Condon, 1999). They studied the role of pHi in the induction of ATR by exposing cells to various external pH (pHo) and to various acids with different pKa. Both the above

two studies revealed that level of ATR depends on pHi at the time of induction rather that pHo. Sriraman (2008) has made similar observations, linking the decrease in pHi to the onset of ATR in streptokinase- producing recombinant *L.*

lactis MG1363. The cytoplasmic concentration of H+ or some metabolite whose concentration is directly dependent on the intracellular H+ concentration stimulates the synthesis of proteins which protect *L. lactis* subsp. c*remoris.*

To understand the genetics of acid tolerance response Rallu *et al*. (2002) adopted a strategy through selection and characterization of acid mutant strains. Based on various phenotypes obtained by acid stress selection they classified four classes of mutants, two of which comprises multi stress resistance strains. The variety of mutant phenotypes obtained suggests that this acidifying bacterium has several acid tolerance responses and/or regulatory networks. Mutants with altered phosphate transport have a mutation in the pstS gene, which belongs to high affinity phosphate transport system and is required at low concentrations of phosphate. They observed that acid resistant phenotype could be of pstS mutant and could be reversed by supplying phosphate to the growth medium. They proposed that pstS leads to lower intracellular phosphate and this may act as signal for acid stress.

Mutants with altered purine metabolism include relA, deoB, hpt, guaA and arl7 (uncharacterized gene). RelA protein is responsible for ppGppp synthesis during the stringent response and is well conserved in Gram-positive and Gram-negative bacteria. DeoB codes for phosphopentamutase which is involved in purine and pyramidine salvage pathways and degradation of nucleosides. Hpt codes for hypoxanthine guanine phosphoribosyl transferase required for conversion of guanine to guanosine monophosphate (GMP). GuaA codes for GMP synthase, an enzyme which catalyses the systhesis of GMP from XMP. All these enzymes alter the levels of either GP pools (guanine phosphate) or ppGppp pools suggesting the importance of these pools in acid resistance. It was proposed that altered GP and ppGppp pools lead to induction of stringent response and thus along with acid develops resistance against other stresses also. Xie *et al*. (2004) found that in *L. lactis* IL1403, deoB gene is upregulated which suggest development of ATR in IL 1403 may be independent of stringent response.

Further, characterization of these mutants through analysis of proteomic data was carried out recently to understand the molecular basis of ATR and its regulation. They identified out of 58 proteins which are deregulated in the mutants, only 6 proteins are found to be common to all three mutants. The adaptation appears to involve multiple genes as indicated by changes in extracted proteins separated by two-dimensional gel electrophoresis. Upon acid shock (pH 4.5 or below), synthesis of 43 proteins is induced. The stationary phase sigma factor, σs, is also induced and is responsible for the induction of seven additional proteins (Leenhouts *et al*., 1995; Sa *et al*., 2012; Mukherjee *et al*., 2015). All these proteins are predicted to be involved in repair of acid pH damage and survival.

The proteins expressed in *L. lactis* in response to acid stress response was also studied by Frees *et al*. (2003). To investigate the expressed proteins they exposed *L. lactis* to conditions of low pH and used two-dimensional gel electrophoresis to follow how protein expression changes with the degree of acidification. They found that reducing the pH of the growth medium with hydrochloric acid induced the synthesis of a small subset of proteins. The majority of these

proteins were induced both after a minor (pH 5.5) and a major (pH 4.5) reduction in pH. Among the most strongly induced proteins, they identified the oxidative stress proteins superoxide dismutase, SodA and alkylhydroperoxidase as well as the autoinducer synthesis protein, LuxS (Akshy *et al*., 2016; Rahi and Vyas, 2015). They also observed a differential induction of heat shock proteins by low pH as members of the CtsR regulon, ClpE and ClpP were induced at both pH 5.5 and 4.5, while HrcA-regulated chaperones, GroEL, GroES, DnaK and GrpE were induced only at pH 4.5.

In addition, two proteins were identified which are repressed by low pH are *L. lactis* HPr protein of the phosphoenolpyruvate sugar phosphotransferase system and the trigger factor known to participate in the folding of newly synthesized polypeptides. Kilstrup *et al*. (1997) identified expression of Hsp14 and Hsp17 during as a part of acid shock response.

Development of ATR causes up-regulation of surface protease HtrA, which causes degradation of recombinant streptokinase in secretory expression system of *L.lacts*. Suppression of ATR causes down regulation of HtRA, which reduces the degradation of streptokinase. The suppression of the ATR was achieved by regulating the buffering capacity of the medium used for culture (Sriraman and Jayaraman, 2006; Gupta *et al*., 2014). In this study M17 medium was used for studying the expression of streptokinase in *L. lactis* MG1363. It was found that in M17 medium with initial glucose concentration 5g/l, medium pH decreased from initial pH of 7.3±0.1 to 5.6±0.1 due to the production of lactic acid, which in turn developed ATR in *L. lactis*. The development of ATR reduced the specific activity of streptokinase by degradation of the functionally active form. The buffering capacity of medium was increased by increasing the concentration of sodium β-glycerophosphate (a buffering agent of M17 medium) in the medium (Chakraborty *et al*., 2015; Chauhan *et al*., 2017).

Bioreactor strategies for production of recombinant proteins in *L. lactis*

Several strategies have been attempted for the enhancement of recombinant protein production in bacterial systems. Recombinant protein production can be significantly increased through use of high cell density culture systems, which includes fed-batch and total cell recycle operations. High cell density culture has the added advantage of increased cost effectiveness, reduced culture volume, enhanced downstream processing, reduced wastewater, lower production costs and a reduced investment in equipments.

There are many studies on high cell density fermentations with wild-type *L. lactis* cultures. Continuous separation of inhibitory metabolites by cross-flow filtration has been employed for high-concentration cultivation of homo-fermentative lactic acid bacteria in order to improve the productivity of cell-mass. The fermentation system allowed continuous removal of lactate, an inhibitory product, while retaining cells completely in the fermentor. A high cell concentration of 141 g/l with 94% viability after 238-h cultivation was achieved by filtering massive amounts of a diluted substrate solution containing 6g/l glucose and 2 g/l yeast extract. Both the yield and productivity of cells were also increased by controlling the feeding of the diluted fresh

medium and filtering the supernatant of the dense cell culture (Suzuki, 1996). By maintaining a low concentration of lactate in the medium, *Streptococcus cremoris* and *Lactobacillus casei* were cultivated to high concentrations of 81.5 and 49.0 g-dry cell weight per liter, respectively, giving cell productivities of 19-fold and 9-fold compared with the corresponding conventional batch cultivations. The time course of the cultivation with cross-flow filtration was predicted on the basis of the assumption that specific growth rate can be expressed as a function only of lactate concentration in the medium (Kobayashi Takeshi *et al*., 1987; Sivakumar *et al*., 2011; Tanwar *et al*., 2012).

To optimize high cell density culture several studies are required on the effects of growth medium, culture conditions and nutrient feeding strategies on recombinant protein production. Other than batch cultures, few studies are available in literature on bioreactor strategies for recombinant protein production with *L. lactis*. Some studies on fed-batch and cell recycle cultures are reported below.

Very few studies have been carried for optimization of recombinant protein production in *Lactococcus lactis*. Production of recombinant green fluorescent protein was optimized in *L. lactis* IL1403 in fed batch culture. In that study a set of surface response experiments were carried out to derive a optimal set of conditions for expression of green fluorescence protein, which includes pH, temperature, hemin concentration, concentration of Nisin inducer per cell and the time of induction. Compared to the traditional batch process the experimental optimal conditions in fed batch culture gave 4.9 times the cell density, 1.6 times the protein per cell mass and 8 times the total protein concentration.

The extremophilic α-glucosidase was cloned in *L. lactis* and correctly folded despite being expressed at a lower temperature. Maximum activity corresponded to 40 U/l in static cultures. The protein yield was further improved by optimizing fermentation and reached 600 U/l in batch mode. Microfiltration led to an even higher enzyme production of 850 U/l as a result of increased biomass. The overall production of α-glucosidase using the engineered *L. lactis* strain in microfiltration fermentation was 1,000-fold higher than obtained using the wild-type.

Streptokinase

Streptokinase, a 47 kDa protein, is used as a thrombolytic agent in the treatment of acute myocardial infarctions and pulmonary embolism. Of the three major thrombolytic agents, the microbial plasminogen activator streptokinase mediates proteolytic conversion of the inactive proenzyme plasminogen to its active form plasmin. Streptokinase was first described as a thrombolytic drug (Tillett and Garner, 1933) and it was introduced as a therapeutic agent for acute myocardial infarctions more than four decades ago. It is included in the WHO model list of essential medicines and is used as a potent thrombolytic agent. The efficacy and clinical implications of streptokinase potentially depends on its dosage (Hermentin *et al*., 2005). Investigations on the different commercially available streptokinase formulations have shown a significant discrepancy between the label claim and the measured streptokinase activity (Couto *et al*., 2004; Hermentin *et al*., 2005). This necessitates the design of a robust production and efficient purification processes for recombinant streptokinase.

Conclusions

There are inherent disadvantages in using anaerobic systems such as *L. lactis* for recombinant protein production. Cell density and specific productivity are poor compared to aerobic organisms such as *E. coli* due to the poor energy availability for cell growth and protein synthesis. Moreover, organisms such as *E. coli* have been extensively studied and genetically manipulated for expressing high amounts of recombinant proteins. Bioprocess strategies have also been fine-tuned with recombinant *E. coli* in order to achieve high cell densities and high volumetric productivity. Such extensive studies have not been carried out with *L. lactis*, especially at the bioprocess level, to fine-tune its productivity. This work attempts to address this lacuna using the P170 expression system.

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