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Review

Mannheimia haemolytica growth and leukotoxin production for vaccine manufacturing — A bioprocess review

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ABSTRACT

Mannheimia haemolytica leukotoxin (LKT) is a known cause of bovine respiratory disease (BRD) which results in severe economic losses in the cattle industry (up to USD 1 billion per year in the USA). Vaccines based on LKT offer the most promising measure to contain BRD outbreaks and are already commercially available. However, insufficient LKT yields, predominantly reflecting a lack of knowledge about the LKT expression process, remain a significant engineering problem and further bioprocess optimization is required to increase process efficiency. Most previous investigations have focused on LKT activity and cell growth, but neither of these parameters defines reliable criteria for the improvement of LKT yields. In this article, we review the most important process conditions and operational parameters (temperature, pH, substrate concentration, dissolved oxygen level, medium composition and the presence of metabolites) from a bioprocess engineering perspective, in order to maximize LKT yields.

Contents

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1. Introduction

Bovine respiratory disease (BRD) is economically the most important disease in the cattle industry although it also affects other wild and domestic ruminants [1,2,3,4]. The high morbidity and up to 50% mortality result in considerable losses [5] often approaching $US 1 billion per year in the US cattle industry alone [6,7,8,9].

BRD is a complex multifactorial disease causing a severe form of pneumonia. A BRD outbreak typically occurs after transportation to feedlots, hence the common name for the disease is 'shipping fever' [4,10]. Although the mechanism of infection and the complex interactions among the host, pathogen and environment are not fully understood, *Mannheimia haemolytica* leukotoxin (LKT) is the predominant virulence factor [6,11,12,13,14,15].

M. haemolytica is a Gram-negative, facultative anaerobic, non-motile, opportunistic pathogen [16]. As a commensal organism of the upper respiratory tract and nasopharynx of healthy ruminants, it can colonize the lower respiratory tract of stressed or immunocompromised animals and overcome their innate immunity, causing pneumonia [7,16]. LKT is a 105-kDa, soluble, heat-labile protein that belongs to the repeat-in-toxin (RTX) family, and it has a dose-dependent effect. At low concentrations, LKT induces bovine cells to undergo a respiratory burst and degranulation thus causing inflammatory cytokine production. At higher concentrations, LKT induces apoptosis and the formation of transmembrane pores, the latter resulting in necrosis and the breakdown of the pulmonary immune system [8,13,17]. LKT is closely related to *Escherichia coli* α-hemolysin and is similarly encoded by a four-gene polycistronic operon (*lkt*CABD). The *lkt*A gene encodes the inactive proLKT protein, whereas *lkt*B and *lkt*D encode proteins that promote secretion [18,19,20,21], and *lkt*C encodes the enzyme that activates LKT by acylation [19]. The expression and activation of LKT has been comprehensively reviewed [12,19,22,23,24,25,26,27].

More than 20 *M. haemolytica* serotypes, subdivided into two biotypes (A and T), have been identified thus far, revealing a high degree of amino acid sequence diversity for LKT due to the complex gene mosaic structure [3,8,28,29,30]. The most relevant forms from a veterinary perspective are biotype A serotype 1 in cattle and biotype A serotype 2 in sheep [28,31]. The treatment of BRD typically involves aggressive antimicrobial therapy, combined with improved feedlot management and vaccination to prevent further outbreaks [6,8]. Although antimicrobials are widely used, they are becoming less effective due to the spread of antibiotic resistance [2,8,15,32]. The demand for BRD vaccines is therefore rising, and currently-available vaccines based on LKT as the predominant antigen are highly effective [33,34,35,36]. The role of several other virulence factors of *M. haemolytica* such as the capsule, outer membrane proteins (e.g. PIpE), neuraminidase, adhesins, and lipopolysaccharides have also been investigated for vaccine formulation [8,28,37,38,39,40]. A PIpE-LKT fusion protein as antigen showed a significant protection against a bacterial challenge [39,41,42]. Nevertheless, LKT provided as *M. haemolytica* supernatant is still the most relevant and successfully applied antigen for vaccination. However, the yields of LKT are often low [43,44] and it is unclear whether the rising demand for the vaccine can be met by current processes. This review article therefore focuses on the optimization of LKT yields in *M. haemolytica* from a bioprocess engineering perspective. Major process parameters such as temperature, dissolved oxygen concentration and media composition are considered based on the hypothesis that *M. haemolytica* experiences comparably dramatic changes in its physical environment during the course of infection.

2. The expression profile of LKT

LKT expression should occur during the log phase of cell growth but the precise expression profile remains uncharacterized [12,19,45,46,47,48]. Moreover, higher growth rates and more biomass do not necessarily lead to higher LKT yields [44,49,50]. However, previous investigations often focused on *M. haemolytica* growth and LKT activity, and there is little correlation between the total amount of LKT in the culture supernatant and LKT activity [45]. One potential reason for this is the strong dependence of LKT activity on temperature. The complex and non-standardized preparation of samples for current LKT activity assays can lead to the rapid thermal inactivation of LKT, resulting in high standard errors [51]. Furthermore, there is high strain-dependent variability in terms of optimum LKT expression, making it difficult to generalize previous investigations [30,43,47,48,49]. As a result, cell growth rate and LKT activity are not strictly reliable as criteria for the optimization of LKT expression, and a clear differentiation among optimal cell growth, LKT activity and LKT expression is therefore necessary. The Enzyme-linked Immunosorbent Assay (ELISA) is the most common and well established method to quantify LKT expression [38,52].

3. Process and kinetic parameters

The available data concerning *M. haemolytica* media and process requirements for cell growth and LKT production are limited and often contradictory (Table 1). However, *M. haemolytica* experiences dramatic changes in its physical environment during the course of infection, including changes in temperature, oxygen levels and nutrient availability. Therefore, critical factors such as media composition, pH, dissolved oxygen, inoculum density and their effects on cell growth and LKT expression are discussed in more detail below, including the impact of acetic acid as the major metabolic byproduct (Table 2, Table 3).

3.1. Medium requirements and supplements

LKT production usually involves a two-stage batch process including a change in the medium composition [44,47]. The most common media for LKT production are brain heart infusion (BHI) broth and chemically

Table 1
Critical medium components affecting *M. haemolytica* cell growth, LKT activity and LKT expression.

		Inhibitory/sub-optimal	Beneficial/essential
Complex media supplements	Growth	N/A	Yeast extract
	LKT expression	Yeast extract	N/A
	LKT activity	N/A	BSA, FCS
Carbon source	Growth	Galactose, glycerol, sucrose, lactate	Glucose
	LKT expression	N/A	N/A
Amino acids	Growth	L-Methionine	L-Alanine, L-Isoleucine
	LKT expression	The absence of amino acids	N/A
Vitamins	Growth	N/A	Calcium pantothenate, nicotinamide, thiamine
	LKT expression	N/A	N/A
Trace elements	Growth	BSA + Fe^{3+}+Mg^{2+}, Ca^{2+}	Fe^{3+}, Mg^{2+}
	LKT expression	BSA + Fe^{3+}+Mg^{2+}, Ca^{2+}	Mn^{2+}+Fe^{3+}
	LKT activity	BSA + Fe^{3+}+Mg^{2+}, Ca^{2+}	N/A

Table 2
Critical process conditions affecting *M. haemolytica* cell growth, LKT activity and LKT expression.

		Non-permissive	Sub-optimal	Optimal
pH	Growth	≤6.5, ≥8.8	≤7.1, ≥7.9	7.2–7.8
	LKT activity	≤6.2	≤6.7, ≥6.9	6.8
	LKT expression	N/A	≤7.2[a]	7.3–8.0[a]
DO	Growth	N/A	Non-aerated	Aerated
	LKT activity	N/A	Non-aerated	Aerated
	LKT expression	N/A	Non-aerated	Aerated
Temp.	Growth	≤15.3°C, ≥43.2°C	≤36°C, ≥41°C	37–40°C
	LKT activity	N/A	N/A	N/A
	LKT expression	≤30°C	≤36°C, ≥41°C	37–40°C

[a] Qualitative SDS-PAGE analysis.

defined RPMI-1640 medium, which is often supplemented with fetal calf serum (FCS) or bovine serum albumin (BSA) [44,47]. However, FCS and BSA enhance LKT activity more than LKT expression [43,44,47,53,54,55,56,57].

3.1.1. Carbon source

Various carbon sources have been used with *M. haemolytica* but glucose is the substrate of choice (Table 1, Table 3) [56,58]. However, glucose favors the production of large amounts of acetic acid as a metabolic byproduct, and the resulting drop in pH inhibits cell growth [44,58]. Up to 87% of the carbon derived from glucose can be channeled into acetic acid production once the glucose concentration rises above a certain threshold [44]. Although the critical glucose and acetic acid concentrations in *M. haemolytica* are unknown, the carbon overflow mechanism is probably similar to that reported in *E. coli* [44], which has a glucose threshold of 30 mg L^{-1} and an acetic acid inhibition constant (k_i) of 9 g L^{-1} [59,60].

Alternative carbon sources that support the efficient growth of *M. haemolytica* include D-xylose, D-mannitol, D-ribose, D-sorbitol and fructose, but these also promote the synthesis of acetic acid. In contrast, galactose, glycerol, sucrose and lactate do not promote the synthesis of acetic acid as much as other carbon sources, but they also result in slow growth rates [58].

3.1.2. Amino acids

The amino acid requirements of *M. haemolytica* are strictly limited to L-amino acids. Alanine and isoleucine are essential for rapid cell growth, whereas hydroxyproline, serine and either threonine or tryptophan are completely dispensable, and methionine even has a small inhibitory effect [61]. Furthermore, LKT is not produced under amino acid limiting conditions [56]. Therefore, amino acid supplements can achieve a significant boost in LKT expression levels especially when defined or semi-defined media are used. This predominantly reflects the increase in cysteine and glutamine levels, revealing a possible bottleneck for further optimization [44,49].

In contrast to the reports summarized above, van Rensburg and du Preez [49] found that when yeast extract is used as the amino acid source, LKT production is favored under amino acid limiting conditions compared to carbon limiting conditions. Nevertheless, du Preez [44] found that although yeast extract promotes cell growth, it also reduces the amount of LKT produced per cell because precursors and energy are committed to the accumulation of biomass, and this may explain the results presented by van Rensburg and du Preez [49]. The essential role of amino acids is supported by two hypotheses: (i) cysteine is essential for amino acid metabolism in *M. haemolytica* because this species cannot reduce sulfate for incorporation into sulfur-containing amino acids, and (ii) amino acids can also serve as a nitrogen source for *M. haemolytica* [44,56].

3.1.3. Vitamins

Only calcium pantothenate, nicotinamide, and the monophosphate or pyrophosphate of thiamine are thought to be essential for the optimal growth of *M. haemolytica*, but increasing the initial vitamin concentration does not appear to boost LKT expression [44,56,58]. Other common components such as biotin, folic acid, p-aminobenzoic acid, pyridoxine, riboflavin, hemin and oleic acid do not appear to affect cell growth [58,61].

3.1.4. Trace elements

Iron may be a key trace element required for the growth of *M. haemolytica* and this mineral is physiologically available as bovine transferrin with an iron level regulated by receptor expression [37,62].

Table 3
Overview of published process settings and yield coefficients for different *M. haemolytica strains.*

Strain	H-44L	H-44L	Biotype A, serotype 1[a]		OVI-1		OVI-1				OVI-1
Medium	Casein	CDM	RPMI 1640	BHI	BHI + Yeast extract		BHI	RPMI 1640	SDM[b]	SDM[c]	SDM[b]
Carbon source	Glc	Gal/Glc	Glc	Glc	Glc		Glc	Glc	Glc	Glc	Glc
Equipment	SF	SF	SF	SF	SF	BR	BR	BR	BR	BR	BR
Process mode	Batch	Batch	Batch	Batch	Batch	Batch	Batch	Batch	Batch	Batch	Conti.
T [°C]	37	37	37	37	37	37	37	37	37	37	37
pH	7.4[d]	7.4[d]	6.8[d]	6.8[d]	7.3[d]	7.3[d]	7.3	7.3	7.3	7.3	7.3
DO [%]	N/A	N/A	N/A	N/A	N/A	≥30	≥30	≥30	≥30	≥30	≥30
n [rpm]	150	150	120	120	200	400–550	400–550	400–550	400–550	400–550	400–550
t [h]	12	25	4.0	4.0	3.5	3.0	3.5	5.5	4.5	9.5	N/A
$OD_{575/600}$	0.52	0.60	0.621	0.594	N/A	N/A	N/A	N/A	N/A	N/A	N/A
$c_{Biomass}$ [g/L]	N/A	N/A	N/A	N/A	1.6[e]	2.3[e]	1.80	0.41	1.62	0.88	1.08
μ_{max} [h^{-1}]	N/A	N/A	0.56	1.03	1.19	1.15	1.56	0.62	1.25	0.71	1.0[f]
$Y_{x/s}$	N/A	N/A	N/A	N/A	N/A	N/A	0.56	0.20	0.52	0.17	0.60
c_{AA} [g/L]	N/A	N/A	N/A	N/A	1.9[e]	2.08	1.80	1.73	1.40	2.41	1.05
c_{LKT} [g/L]	N/A	N/A	0.04[g]	0.06[g]	N/A	N/A	0.02	0.04	0.07	0.33	0.04[g]
Reference	[58]	[61]	[43]		[70]		[49]				[44]

CDM = chemically-defined medium, SDM = semi-defined medium, Glc = glucose, Gal = galactose, SF = shake-flask, BR = bioreactor, T = temperature, DO = dissolved oxygen, n = agitation, t = cultivation time, $c_{Biomass}$ = biomass concentration, μ_{max} = maximum growth rate, $Y_{x/s}$ = biomass yield coefficient on glucose utilized, c_{AA} = acetic acid concentration, c_{LKT} = leukotoxin concentration.

[a] Not further specified.
[b] Glucose limited.
[c] Amino acid limited.
[d] No control.
[e] Data obtained from diagram.
[f] Dilution rate.
[g] Rounded to two decimal places.

Iron-supplemented RPMI-1640 medium increased the total biomass and maximum cell growth rate of *M. haemolytica* [43]. Previous investigations have indicated that iron is required for LKT secretion and that iron also induces the expression of the *lkt*CABD operon [18,43,46,49,62,63]. The co-presentation of manganese and ferric iron boosted LKT expression even further [49]. However, data have also been published showing greater *lkt* promoter activity under iron-limiting conditions [50]. This contradictory finding is supported by the regulation of other bacterial toxins, including outer membrane proteins and siderophores, where iron-limiting conditions stimulated their production [62,64,65,66,67].

Depending on which culture medium is used, the addition of 0.001–0.01 M magnesium can increase the total biomass [43,58], whereas the combination of BSA, magnesium and ferric iron resulted in lower biomass accumulation and a steep decline in the LKT yield [43]. Taken together, these data suggest that iron, magnesium and manganese are the most promising trace elements to promote *M. haemolytica* cell growth and LKT expression.

3.2. Process conditions

3.2.1. pH

In complex media, the highest maximum growth rate of *M. haemolytica* is achieved when the initial pH lies within the range 7.2–7.8 [49,58]. At pH values of ≤6.5 or ≥8.8, growth slows down or even stops completely [49,58]. A similar optimum pH range (7.3–8.0) has been reported for LKT expression [46], but the data are based on qualitative SDS-PAGE analysis and the specific production rate per dry weight of cell biomass has not been reported. Within this optimum pH range, *lkt* promoter activity remains constant [50], but if the pH falls below 7.3 there is a reversible decline in LKT expression [46]. Interestingly, the optimum pH for LKT activity is 6.8, which is not within the optimal ranges for either cell growth or LKT expression [43].

Because *M. haemolytica* produces large amounts of acetic acid, pH shifts during cultivation are unavoidable. However, the concentration of phosphate or tris(hydroxymethyl)aminomethane (Tris) required to maintain a constant pH is toxic and therefore inhibits cell growth [58]. Recent efforts have focused on pH-controlled cultivation at pH 7.3 in bioreactor systems, because it is not possible to control the pH adequately in shake-flasks [49]. The production system and scale therefore play key roles in determining the yield of LKT.

3.2.2. Dissolved oxygen

M. haemolytica is a facultative anaerobic bacterium and can therefore grow either in the presence or absence of oxygen. Although the transcription of the *lkt*CABD operon is not dependent on the oxygen level [50] or even favored under anaerobic conditions [23], shake-flask experiments have nevertheless shown that increasing the aeration increases the growth rate and biomass accumulation [68]. Furthermore, well-aerated conditions achieved an almost four-fold increase in LKT production as well as a higher LKT activity compared to non-aerated conditions [45,49]. As a consequence, aerobic conditions are more suitable than anaerobic conditions for the production of LKT.

3.2.3. Temperature

M. haemolytica grows optimally at 37°C, and increasing the temperature up to 40°C does not have an impact on the growth rate [49]. Beyond these ranges, the growth rate decreases and eventually stops at ≤15.3°C or ≥43.2°C [45,49]. The optimum temperature range for LKT expression is 37–40°C. Expression declines at temperatures below 37°C and eventually stops at ≤30°C [45,46]. Studies of the *lkt* promoter have shown a reversible effect caused by shifting the temperature from 30°C to 42°C [50]. Depending on the individual needs in terms of product quality, the thermolability of LKT must be considered in greater detail.

3.2.4. Inoculum density

The total biomass of *M. haemolytica* cultures appears to be independent of the inoculum density at $OD_{575nm} = 0.02$ and $OD_{650nm} = 0.4$ [47,58]. Even inoculum densities of ≤10 cells mL^{-1} can achieve sufficient biomass concentrations, but the lag phase is longer when the inoculum density is this low, resulting in an extended cultivation period [58]. Increasing the inoculum density causes a slight decrease in the maximum growth rate (calculated from published data) [47], which may reflect early nutrient limitations in the RPMI-1640 medium. Depending on the production volume and the required product quality, it may be necessary to use a sub-optimal inoculum density. High inoculum densities reduce the overall cultivation time and therefore also the degree of LKT inactivation, whereas low inoculum densities are more suitable for seed scale-up.

4. Conclusion and outlook

This review summarizes the knowledge about bioprocess requirements for *M. haemolytica* cell growth and LKT production and emphasizing the importance and possibilities to further optimize the LKT production process for vaccine manufacturing. Previous investigations have focused mainly on LKT activity and cell growth, which are not strictly related to LKT expression. The considerable *M. haemolytica* strain variability may explain some of the contradictory results, and strain-specific optimization is therefore recommended.

Acetic acid is the major metabolic byproduct of LKT production in *M. haemolytica* and the amount of acetic acid produced often exceeds the amount of accumulated biomass. This is because up to 87% of the carbon from glucose can be channeled into the carbon overflow metabolism, resulting in the accumulation of acetic acid and a significant drop in pH. Interestingly, the effect of pH-controlled and uncontrolled conditions have not yet been compared. Nevertheless, the large amount of acetic acid produced under optimal conditions in batch cultures is likely to limit *M. haemolytica* cell growth and thus LKT production. Although a pH-controlled process would achieve better cell growth, the accumulation of acetic acid causes two further problems: (i) large amounts of ATP are required to expel protons and avoid the acidification of the cytoplasm [49], and (ii) large amounts of an alkaline solution would be needed to maintain a constant pH, which increases the osmolarity of the medium. No studies have yet been carried out to investigate the impact of osmolarity, and detailed investigations are necessary to determine the effect of acetic acid and its concomitant effects on *M. haemolytica* cell growth and LKT production. The control of acetic acid production may provide a key to optimizing the LKT yield. One potential approach would involve the modulation of the carbon overflow metabolism, which can be achieved in *E. coli* by controlling the oxygen level. Bioreactors are therefore preferable for *M. haemolytica* cultivation because they allow the dissolved oxygen and pH to be controlled simultaneously. Nevertheless, the most promising approach is a carbon limited fed-batch process to favor biomass accumulation while minimizing acetic acid production. A better understanding of the metabolic requirements of *M. haemolytica* is necessary to optimize the feed in a glucose-limited fed-batch process.

In addition to these physical parameters, the cultivation medium has a significant effect on *M. haemolytica* cell growth and LKT production. The nutritional requirements of *M. haemolytica* are not understood in detail, and the commonly-used RPMI-1640 medium was designed for mammalian cells. Optimization may require the absence of ferric iron, as well as low concentrations of glucose, magnesium and amino acids. More efficient LKT production and/or biomass accumulation is possible depending on which supplements are provided. Furthermore, industrial process development would also need to consider the interactions between upstream production and downstream purification, e.g. complex supplements and more biomass can make the purification of LKT more challenging and expensive [43,53,69].

Conflict of interest

The authors declare that they have no conflict of interest.

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