J Ind Microbiol Biotechnol (2008) 35:611–618
DOI 10.1007/s10295-008-0324-y

ORIGINAL PAPER

Kinetics of growth and leukotoxin production by *Mannheimia haemolytica* in continuous culture

James C. du Preez · Eugéne van Rensburg · Stephanus G. Kilian

Received: 15 January 2007 / Accepted: 25 January 2008 / Published online: 19 February 2008

Abstract The growth and product formation kinetics of the bovine pathogen *Mannheimia (Pasteurella) haemolytica* strain OVI-1 in continuous culture were investigated. The leukotoxin (LKT) concentration and yield on biomass could substantially be enhanced by supplementation of a carbon-limited medium with an amino acid mixture or a mixture of cysteine and glutamine. Acetic acid was a major product, increasing to 1.66 g l^{-1} in carbon-limited chemostat culture at intermediate dilution rates and accounting for more than 80% of the glucose carbon, whereas in amino acid-limited cultures high acetic acid concentrations were produced at low dilution rates, suggesting a carbon-overflow metabolism. The maintenance coefficients of carbon-limited and carbon-sufficient cultures were 0.07 and 0.88 mmol glucose g^{-1} h^{-1}, respectively. LKT production was partially growth-associated and the LKT concentration was maximised to 0.15 g l^{-1} and acetic acid production minimised by using a carbon-limited medium and a low dilution rate.

Keywords *Mannheimia haemolytica* · Leukotoxin · Chemostat · Kinetics · Acetic acid

J. C. du Preez (✉) · E. van Rensburg · S. G. Kilian
Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, P.O. Box 339, Bloemfontein 9300, South Africa
e-mail: dpreezjc.sci@ufs.ac.za

Introduction

Mannheimia haemolytica produces a leukotoxin (LKT) that has a specific affinity for bovine alveolar macrophages and leukocytes. LKT is considered the main virulence factor of fibronecrotizing pneumonia of cattle and sheep, also known as pneumonic pasteurellosis or shipping fever [3, 32]. A *M. haemolytica* culture supernatant vaccine based on the LKT toxoid is effective against the disease [4, 8, 27] because of its strong immunogenicity and lack of serotype specificity [17, 20, 28]. However, challenge trials in cattle revealed that vaccination with recombinant LKT (rLKT) alone failed to provide protection against the development of clinical symptoms, which demonstrated the complexity of protective immunity against this respiratory disease [5]. Thus, the culture supernatant vaccine, of which LKT is a key component, proved more efficient than the use of pure rLKT. One drawback in the preparation of a culture supernatant vaccine is that the toxin is usually produced at a very low concentration in vitro [26, 30].

The growth characteristics of *M. haemolytica* are poorly quantified. Acidification of the culture broth due to the growth of *M. haemolytica* has been reported, but the acid produced was not identified [11, 34]. The influence of nutrients on LKT production by this nutritionally fastidious bacterium has been investigated [9, 11, 30]. The most widely used culture media are RPMI 1640 and brain heart infusion broth, often supplemented with bovine foetal serum or albumin, but it has been reported that such supplements had no significant effect on LKT production [32].

This paper is the first detailed report on the growth and product formation kinetics of *M. haemolytica*, using continuous cultures to facilitate specific nutrient limitations during steady-state growth conditions. We report on the

effects of various nutrients on the culture performance, show that *M. haemolytica* produced copious amounts of acetic acid in aerobic culture and that LKT production could be substantially enhanced by the nutrient formulation and selection of the dilution rate. These findings have bearing on the production of LKT in submerged culture for use in immunoprophylaxis.

Materials and methods

Bacterial strains

Mannheimia haemolytica strain OVI-1, a fresh, untyped isolate from the lung of a calf that succumbed to shipping fever pneumonia was provided by the Onderstepoort Veterinary Institute, Gauteng Province, South Africa.

Cultivation procedure

Mannheimia haemolytica was routinely grown at 35 °C on Biolab brain heart infusion (BHI) agar plates (Merck, Darmstadt, Germany) at pH 7.4. Erlenmeyer flasks (250 ml) containing 50 ml culture medium were inoculated from 36 h BHI agar plates and incubated for 9 h at 37 °C on a rotary shaker at 200 rpm. A 5 ml aliquot of the above pre-inoculum was transferred to a 250 ml shake flask containing 50 ml of the same medium and incubated for 3 h at 37 °C prior to being used to inoculate the bioreactor vessel to 0.2 absorbance units at 690 nm. Medium feed was started when the culture reached the late exponential growth phase.

A 2-l Multigen F-2000 bioreactor (New Brunswick Scientific, Edison, NJ, USA) controlled at 37° C and pH 7.3 (±0.2) with 3 M KOH and equipped with two disk turbine impellers, an exhaust gas condensor, a pH electrode (Mettler Toledo, Halstead, UK) and a polarographic oxygen probe (Ingold AG, Urdorf, Switzerland) was used. Peristaltic pumps (Watson Marlow Ltd, Falmouth, UK) fitted with silicone rubber tubing were used to maintain the culture volume at 0.74 l. The dissolved oxygen tension was maintained above 30% of saturation by using an aeration rate of 0.6 l min^{-1} and manual adjustment of the stirrer speed between 400 and 550 rpm. A steady state was confirmed when the deviation in the culture turbidity was less than 2% of the mean for at least three consecutive residence times with no upward or downward trend. At dilution rates (D) approaching the maximum specific growth rate (μ_{max}), up to ten residence times were allowed to ensure steady-state conditions. The μ_{max} value was derived from the slope of the wash-out curve obtained when the dilution rate was incrementally increased to a value greater than wash-out dilution rate.

Culture media

The basal medium used in continuous culture experiments contained (per l) 0.5 g citric acid monohydrate, 3.0 g NaCl, 3.4 g KH_2PO_4, 0.3 g $MgSO_4{\cdot}7H_2O$, 3.0 g $(NH_4)_2SO_4$, 0.02 g $CaCl_2{\cdot}2H_2O$ (all Saarchem; Merck, Wadeville, South Africa), 0.0175 g inositol (BDH Chemicals, Poole, England) and 1.0 ml of a 1,000-fold stock solution of vitamins containing (per l) 0.2 g biotin, 1.0 g nicotinamide, 1.0 g pyridoxine HCl, 1.0 g thiamine HCl, 1.0 g folic acid, 0.2 g riboflavin, 10 ml of a stock solution of 0.5 g cyanocobalamin l^{-1} (all Sigma, St Louis, MO, USA), 0.25 g Ca-pantothenate (Merck) and 1.0 g *p*-amino benzoic acid (Hopkin and Williams, Essex, England), adjusted to pH 7.5. Whereas the carbon-limited medium (designated SDM1) consisted of the above basal medium supplemented with 10.0 g yeast extract (Biolab) and 3.0 g glucose l^{-1} (Saarchem), the amino acid-limited medium (designated SDM2) comprised the above basal medium supplemented with 4.0 g yeast extract and 10.0 g glucose l^{-1}. In some experiments the culture medium was supplemented with 1.0 ml of a 1,000-fold concentrated stock solution of trace elements containing (per l) 14 g $FeSO_4{\cdot}7H_2O$ (Merck), 3.0 g $MnSO_4{\cdot}H_2O$, 5.0 g $ZnSO_4{\cdot}7H_2O$, 0.5 g $CuSO_4{\cdot}5H_2O$, 0.5 g $CoCl_2{\cdot}6H_2O$, 0.5 g $Na_2MoO_2{\cdot}2H_2O$, 0.1 g KI, 1.0 g H_3BO_3 and 1.0 g $Al_2(SO_4)_3{\cdot}18\ H_2O$ (all Saarchem) dissolved in 0.05 M HCl. Solutions of glucose, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $(NH_4)_2SO_4$ and $CaCl_2{\cdot}2H_2O$ were autoclaved separately at 121 °C, whereas inositol and vitamin solutions were filter-sterilised. The complete medium had a final pH of 5.8. A 0.2 ml volume of a 1:1 mixture of Voranol P2000 Polyol and Durapol 3000 (Industrial Urethanes, Edenvale, South Africa) were added as antifoaming agents.

In some experiments the medium was supplemented with one of the following L-amino acid solutions, made up as a 100-fold concentrated stock solution in 0.3 M NaOH, with the exception of cysteine that was separately prepared in 0.1 M HCl. Amino acid mixture A comprised (per l) 5.0 g alanine, 5.0 g arginine, 5.0 g asparagine, 2.0 g aspartic acid, 5.0 g cysteine, 2.0 g glutamic acid, 5.0 g glutamine, 1.0 g glycine, 1.5 g histidine, 5.0 g isoleucine, 5.0 g leucine, 4.0 g lysine, 1.5 g methionine, 1.5 g phenylalanine, 2.0 g proline, 3.0 g serine, 2.0 g threonine, 0.5 g tryptophan, 2.0 g tyrosine and 5.0 g valine. Amino acid mixture B was a four-fold concentrate of mixture A. All amino acids were from Sigma except phenylalanine (Fluka Chemie, Buchs, Switzerland) and glycine (Merck).

Analytical procedures

Culture turbidity was measured with a Photolab S6 photometer (WTW, Weilheim, Germany) at 690 nm. The dry biomass concentration was gravimetrically determined by washing and drying quadruplicate samples overnight at 105 °C to constant mass. Acetic acid was determined after acidification of filtered culture supernatant with formic acid (7.5% v/v, final concentration) with a Hewlett-Packard 5710A gas chromatograph fitted with a glass column (1.5 m × 3.175 mm ID) packed with 80–100 mesh Pora-Pak Q (Waters Associates, Milford, MA, USA) at an oven temperature of 165 °C, an inlet temperature of 200 °C, a hydrogen flame ionization detector at 250 °C and nitrogen carrier gas at 50 ml min^{-1}. Citric acid was determined using a Shimadzu LC-10 HPLC (Shimadzu Corp., Tokyo, Japan) fitted with an SPD-10A detector set at 220 nm and a Hydro-RP column (250 × 4.6 mm ID) with a particle size of 80 Å (Phenomenex, Torrance, CA, USA) and using a mobile phase of 0.05 M KH_2PO_4 (pH 2.7) at a flow rate of 0.8 ml min^{-1}.

Glucose was determined with a glucose assay kit (Sigma), but for concentrations below 1.0 g l^{-1} a D-glucose enzymatic bioanalysis kit (Cat. No. 0716251, R-Biopharm GmbH, Darmstadt, Germany) was used. Culture samples of 10 ml were rapidly aspirated into 30 ml bottles containing 0.5 ml of ice-cold 5 M HCl to inactivate biological activity. The bioreactor exhaust gas composition was determined with a paramagnetic Magnos 6G O_2 analyser and an infrared Uras 10E CO_2 analyser (Hartmann & Braun, Frankfurt, Germany). The distribution of glucose carbon between biomass and acetic acid was based on the volumetric rates of glucose uptake and the production of acetic acid and CO_2, assuming 1 mole of CO_2 produced per mole of acetic acid [10]. The carbon content of the bacterial biomass was estimated using the average biomass formula of $CH_{1.81}O_{0.52}N_{0.21}$ [22].

The LKT concentration in the culture supernatants was determined by an indirect ELISA assay procedure [33], using anti-LKT chicken polyclonal antibody for detection of the LKT and rabbit anti-chicken IgG conjugated to horseradish peroxidase with 3,3′,5,5′-tetramethylbenzidine (Sigma) to provide the colour-forming reaction. The absorbance of test samples was related to protein concentration using the method of Karpinski et al. [14].

Curve fitting was done by non-linear regression using SigmaPlot (Jandel Scientific Software, San Rafael, CA, USA). The steady-state values of the limiting substrate (s) and dry biomass concentration (x) were modelled using transformations of the Monod model incorporating the maintenance coefficient (m) [23]:

$$s = \frac{k_s(D + mY_G)}{\mu_{max} - mY_G - D} \quad (1)$$

$$x = \frac{DY_G(s_r - s)}{D + mY_G} \quad (2)$$

where k_s is the Monod saturation constant, s_r the lmiting substrate (glucose) concentration in the feed reservoir and Y_G the "true" biomass yield coeficient, determined as the inverse of the slope of the specific rate of glucose uptake (q_s) vs D curve.

Results and discussion

Chemostat cultivation in a carbon-limited medium

In an aerobic chemostat culture of *M. haemolytica* OVI-1 using medium SDM1, the decrease in the steady-state biomass concentrations and $Y_{x/s}$ values at dilution rates below 0.21 h^{-1} might be attributed to the maintenance energy requirement (Fig. 1a, b). A maintenance coefficient (m) of 0.07 mmol glucose g^{-1} h^{-1} was determined by linear regression analysis of the linear portion of the q_s vs D curve in the lower range of dilution rates (Fig. 1c) as the q_s value at a dilution rate of zero [2]. Maintenance coefficients of other bacterial species in glucose-limited cultures ranged from 0.39 to 1.05 mmol g^{-1} h^{-1} [6, 15, 23, 24]. Although the biphasic nature of the q_s vs D curve (Fig. 1c) may have resulted in an inaccurate estimation of m, the use of the above m value in Eqs. (1) and (2) resulted in a reasonable fit of the experimental data at low dilution rates (Fig. 1a). Others also have reported the non-linearity of similar plots [2, 6] and it has been postulated that m may in fact be a simple function of the growth rate [21].

However, the onset of a non-carbon limitation with increasing dilution rate was evident from the decrease in the steady-state biomass concentration and increase in the residual glucose concentration at dilution rates much lower than predicted by the Monod model using a k_s value of 4 mg l^{-1} (Fig. 1a). Whereas at low dilution rates a carbon limitation was demonstrated by the increase in steady-state biomass concentration with an increase in the glucose content of the feed, at high dilution rates this had little effect on the steady-state concentrations of biomass and acetic acid but only resulted in a substantial increase in the residual glucose concentration (not shown). When the steady-state residual glucose concentrations were fitted to Eq. (1), an improbably high k_s value of 345 mg glucose l^{-1} was obtained, indicative of a non-carbon limitation.

The μ_{max} value of *M. haemolytica* OVI-1 ranged from 1.35 to 1.39 h^{-1} and was significantly greater than the values of 1.03–1.19 h^{-1} of other *M. haemolytica* strains grown in brain heart infusion broth [29, 30]. The highest

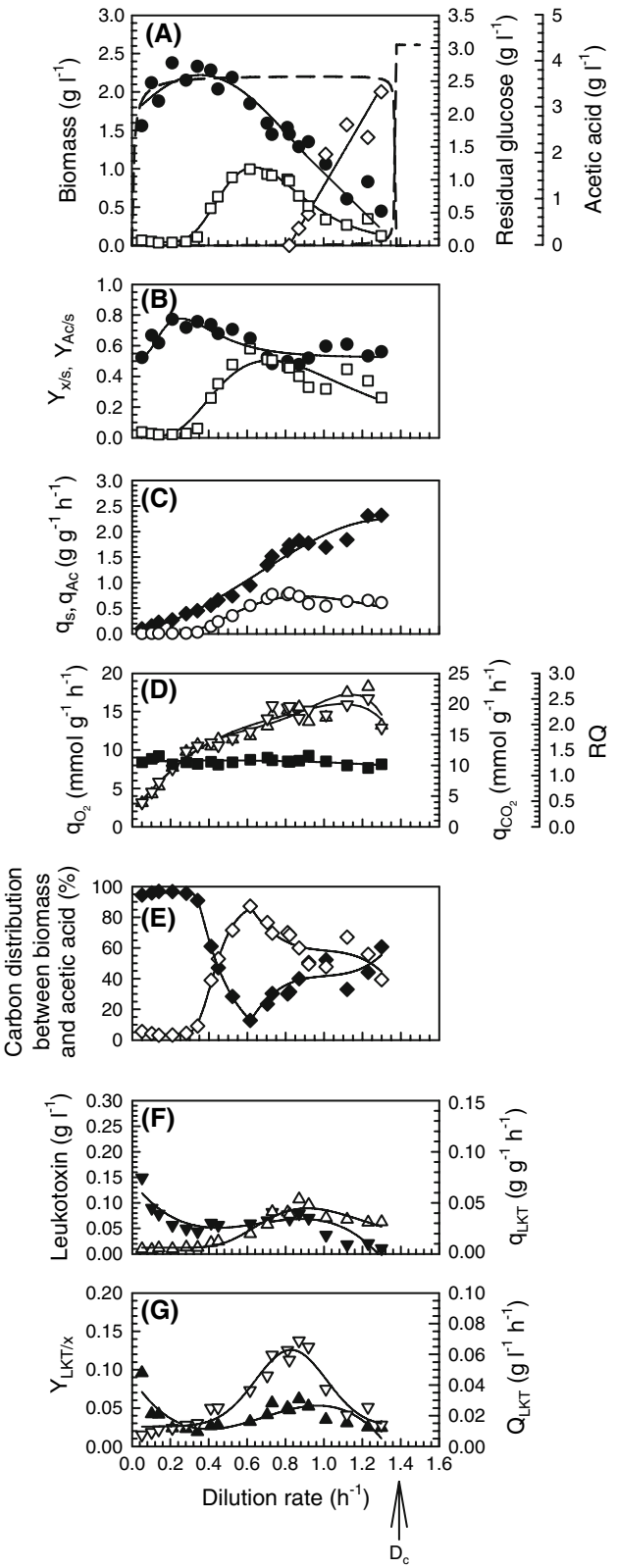

Fig. 1 Steady-state concentrations of **a** dry biomass (*filled circle*), ▶ residual glucose (*open diamond*) and acetic acid (*open square*); **b** the biomass and acetic acid yields on glucose ($Y_{x/s}$, *filled circle*; $Y_{Ac/s}$, *open square*); **c** the specific rates of glucose uptake and acetic acid production (q_s, *filled diamond*; q_{Ac}, *open circle*) plotted as a function of the dilution rate in a culture of *M. haemolytica* strain OVI-1 in SDM1. The specific rates of oxygen uptake (q_{O_2}, *open triangle*), CO_2 evolution (q_{CO_2}, *open inverted triangle*) and respiratory quotient (RQ, *filled square*) are shown in **d** and the relative distribution of glucose carbon to biomass (*filled diamond*) and acetic acid (*open diamond*) in **e**. The steady-state LKT concentration (*filled inverted triangle*) and the specific rate of LKT production (q_{LKT}, *open triangle*) are shown in **f**, whereas **g** shows the LKT yield on biomass ($Y_{LKT/x}$, *filled triangle*) and the volumetric rate of LKT production (Q_{LKT}, *open inverted triangle*). The *broken lines* indicate the biomass and residual glucose concentrations predicted by the Monod model, using a k_s value of 4 mg glucose l^{-1} as stated for *Escherichia coli* [23] and the following experimentally derived values: feed glucose, 3.05 g l^{-1}; μ_{max}, 1.39 h^{-1}; m, 0.073 mmol g^{-1} h^{-1}; Y_G, 0.132 g $mmol^{-1}$

biomass yield coefficient ($Y_{x/s}$) of 0.77 g (g glucose)$^{-1}$ was reached at a dilution rate of 0.21 h^{-1} (Fig. 1b) and the sharp decrease in biomass concentration with increasing dilution rate roughly coincided with an increase in acetic acid production (Fig. 1a). From Fig. 2 it was determined that 0.34 g biomass l^{-1} was produced from non-glucose substrate, resulting in a corrected $Y_{x/s}$ value of 0.42 that agreed with the mean value of 0.444 for microbial growth on glucose [1]. Because the small amount of citric acid included in the medium as chelating agent was not assimilated, the high $Y_{x/s}$ value was probably due to the utilisation of yeast extract in addition to glucose as carbon source.

The highest steady-state LKT concentration of 0.15 g L^{-1} (Fig. 1f) and LKT yield in terms of biomass ($Y_{LKT/x}$, Fig. 1g) were recorded at the lowest dilution rate used (0.051 h^{-1}), whereas the volumetric rate of LKT production peaked at a dilution rate of 0.87 h^{-1} (Fig. 1g). Extrapolation of the specific LKT production rates (q_{LKT}) intercepted the ordinate at a value of 0.0043 g g^{-1} h^{-1} (Fig. 1f), indicating that LKT production was only partially growth-associated.

Whereas low concentrations of acetic were produced at dilution rates below 0.4 h^{-1} (Fig. 1a), the steady-state acetic acid concentration increased dramatically to a maximum of 1.66 g l^{-1} with an increase in dilution rate, with the acetic acid yield coefficient on glucose ($Y_{Ac/s}$) reaching values of 0.47–0.58 between dilution rates of 0.7 and 0.8 h^{-1} (Fig. 1b). Progressively more glucose carbon was used for acetic acid production at dilution rates greater than 0.4 h^{-1} (Fig. 1e), to the extent that at a dilution rate approximately equivalent to 1/2 μ_{max} up to 87% of the carbon from glucose was chanelled to acetic acid.

In carbon-limited continuous cultures of *Escherichia coli*, the aerobic production of acetic acid typically occurred on an increase in dilution rate to above a certain threshold level [12] or at low dilution rates when the feed glucose concentration was increased [7]. Acetic acid production by *M. haemolytica* followed a similar pattern in that the steady-state acetic acid concentration increased dramatically above a dilution rate of 0.4 h^{-1} (Fig. 1a).

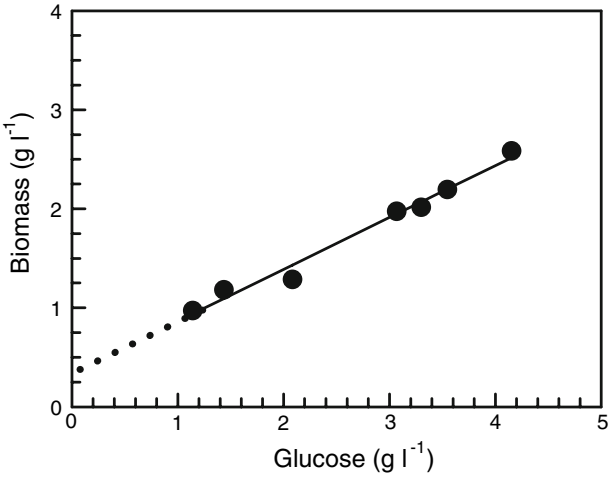

Fig. 2 Steady-state concentrations of dry biomass plotted as a function of the feed glucose concentration in a continuous culture of *M. haemolytica* strain OVI-1 grown in medium SDM1 supplemented with 4.0 g yeast extract at a dilution rate of 0.35 h^{-1}. The intercept on the ordinate indicates the biomass concentration produced from non-glucose carbon

Furthermore, the production of high acetic acid concentrations at low dilution rates in glucose-sufficient continuous culture (Fig. 3a) suggested a carbon overflow metabolism, similar to *E. coli* where the excess carbon flux though the central metabolic pathways was excreted as acetate [13].

Chemostat cultivation in an amino acid-limited medium

A glucose concentration of 15.0 g L^{-1} was used in the amino acid-limited medium SDM2 to ensure that glucose remained in excess. The $Y_{x/s}$ values at low dilution rates were more than three-fold lower than in SDM1 (Fig. 3b), probably due to the high maintenance coefficient of 0.88 mmol glucose g^{-1} h^{-1} determined from the linear part of the q_s vs D curve (Fig. 3c). A high maintenance coefficient under energy (carbon)-sufficient conditions is thought to be due to defective regulation or an uncoupling of the energy metabolism [31].

In contrast to the glucose-limited culture, the highest acetic acid concentration of 4.16 g L^{-1} was produced at the lowest dilution rate and decreased with an increase in dilution rate (Fig. 3a). The RQ remained slightly above unity, which can be expected during aerobic acetic acid production as a result of CO_2 liberation during the dehydrogenation of pyruvate to acetyl∼CoA, followed by acetate production *via* acetyl phosphate at the cost of carbon flow through the oxidative pathways [13, 19]. Calculation of the distribution of glucose carbon revealed that below a dilution rate of 0.5 h^{-1} up to 66% of the carbon from glucose was used for biomass production

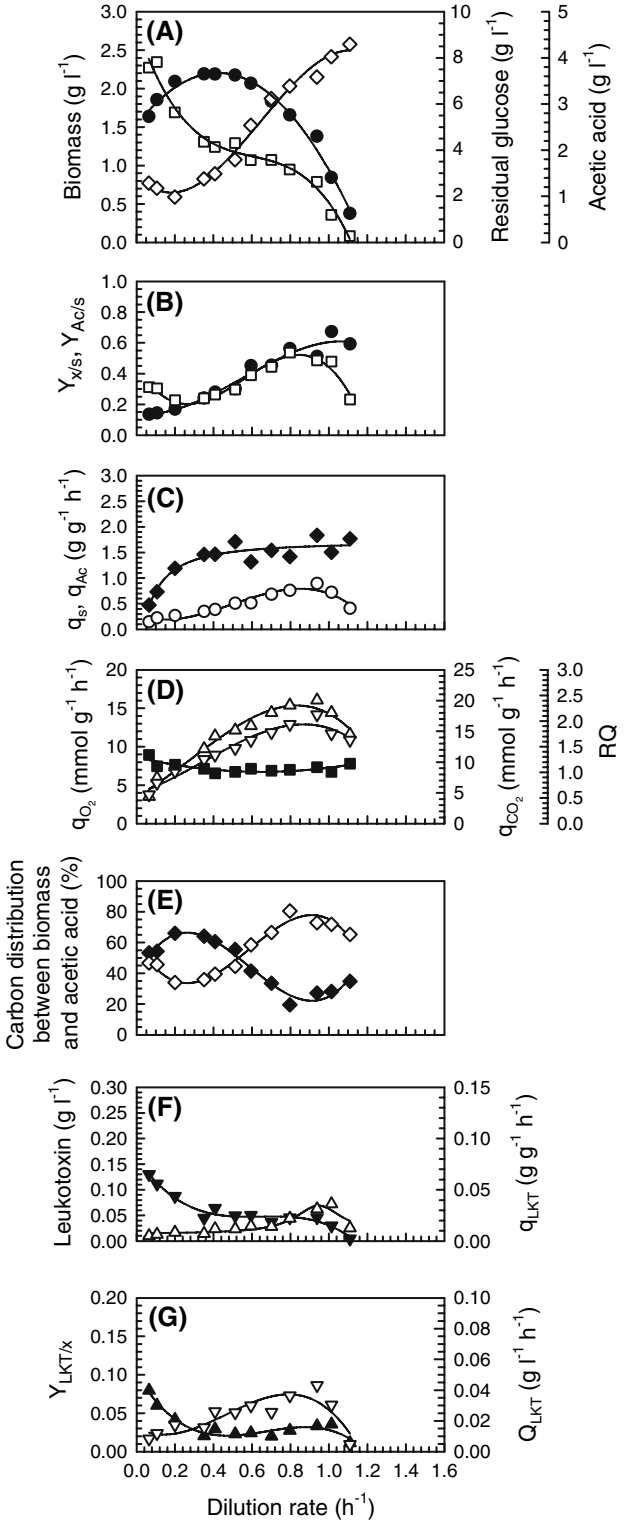

Fig. 3 Steady-state culture parameters of *M. haemolytica* grown in medium SDM2 containing 15 g glucose l^{-1}. Symbols as in Fig. 1

(Fig. 3e), whereas at high dilution rates up to 80% of glucose carbon went to acetic acid production. The highest LKT concentration of 0.13 g l^{-1} and $Y_{LKT/x}$ value were

Table 1 The influence of different nutrient supplements in the feed on the steady-state parameters of a chemostat culture of *M. haemolytica* grown in medium SDM1 at a dilution rate of 1.0 (±0.02 h^{-1})

Nutrient	x[a] ($g\ l^{-1}$)	s[b] ($g\ l^{-1}$)	$Y_{x/s}$	Acetic acid ($g\ l^{-1}$)	$Y_{Ac/s}$	$Y_{Ac/x}$	LKT ($g\ l^{-1}$)	$Y_{LKT/x}$
Control[e]	1.08	1.29	0.60	1.05	0.58	0.98	0.041	0.038
Yeast extract (15 $g\ l^{-1}$)	2.0	0	0.62	1.67	0.52	0.84	0.049	0.024
Vitamin mixture[c]	1.12	1.29	0.63	0.91	0.51	0.81	0.034	0.031
Amino acid mixture A	1.09	1.33	0.52	1.04	0.50	0.95	0.052	0.048
Amino acid mixture B	1.16	0.43	0.45	1.28	0.50	1.10	0.13	0.110
Trace elements solution	0.89	1.21	0.46	0.78	0.40	0.88	0.049	0.055
Cys & Gln (0.4 $g\ l^{-1}$)[d]	1.28	0.77	0.62	1.24	0.60	0.97	0.089	0.070
Gln (0.8 $g\ l^{-1}$) + $Na_2S{\cdot}8H_2O$ (0.25 $g\ l^{-1}$)	1.48	0.31	0.53	1.43	0.51	0.97	0.091	0.062
Cys & Gln (0.4 $g\ l^{-1}$) + trace elements + vitamin mixture[c]	1.30	0.52	0.58	1.24	0.55	0.96	0.135	0.104
Thiamine[e] (0.001 $g\ l^{-1}$)	1.03	1.37	0.54	0.90	0.47	0.87	0.033	0.032

$Y_{x/s}$ Biomass yield coefficient on glucose utilized, $Y_{Ac/s}$ Acetic acid yield coefficient on glucose utilized, $Y_{Ac/x}$ Acetic acid yield coefficient in terms of biomass, $Y_{LKT/x}$ Leukotoxin yield coefficient in terms of biomass

[a] Biomass concentration

[b] Residual glucose concentration

[c] Vitamin solution at double the concentration as described in Materials and methods

[d] L-cysteine plus L-glutamine

[e] Thiamine monophosphate

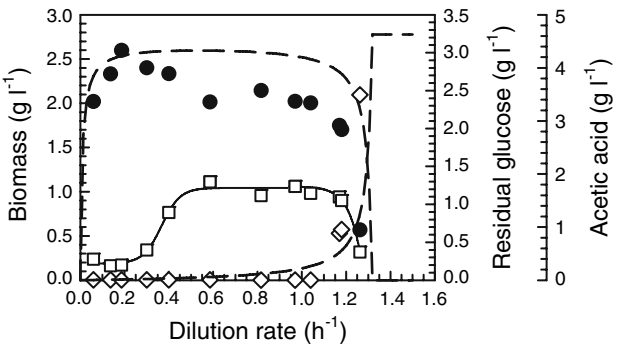

Fig. 4 Steady-state concentrations of dry biomass (*filled circle*), residual glucose (*open diamond*) and acetic acid (*open square*) plotted as a function of the dilution rate in a culture of *M. haemolytica* grown in medium SDM1 supplemented with 15 g yeast extract l^{-1}. The *broken lines* indicate the biomass and residual glucose concentrations predicted by the Monod model, using the following experimentally derived values: feed glucose, 3.24 g l^{-1}; μ_{max}, 1.35 h^{-1}, k_s, 53 mg glucose l^{-1}; m, 0.063 mmol g^{-1} h^{-1}; Y_G, 0.148 g $mmol^{-1}$

obtained at the lowest dilution rate and decreased at the higher dilution rates (Fig. 3f, g).

Effect of nutrient supplements at high dilution rates

To determine the nature of the non-glucose limitation evident when using SDM1 at high dilution rates, further chemostat experiments at a dilution rate of 1.0 h^{-1} were conducted. The apparent growth factor limitation could be abolished by supplementation with yeast extract at 15 g l^{-1} (but without improving LKT production), whereas supplementation with amino acid mixture B or a mixture of cysteine and glutamine, with or without vitamins and trace elements, not only substantially decreased the high residual glucose concentration but also increased the LKT concentration and $Y_{LKT/x}$ value up to three-fold compared to cultures in SDM1 (Table 1). Doubling the concentrations of KH_2PO_4, NaCl, $CaCl_2$, $(NH_4)_2SO_4$ and $MgSO4{\cdot}7H_2O$ in the medium feed had little effect on the culture parameters (not shown), as did the addition of trace elements, amino acid mixture A, a vitamin mixture or thiamine (Table 1), even though the latter was a requirement of *M. haemolytica* strain H44L [35].

When using a yeast extract concentration of 15.0 g l^{-1} (Fig. 4) a more realistic k_s value of 53 mg glucose L^{-1} was determined. This k_s value was still high compared to recent reports of k_s values ranging from about 0.03–0.18 mg l^{-1} for carbon-limited growth of *E. coli* on simple sugars such as glucose, galactose, maltose, ribose and fructose [16, 18, 25]. The maintenance coefficient of 0.063 mmol g^{-1} h^{-1} was similar to that recorded in SDM1 with 10 g yeast extract l^{-1}. However, at the higher dilution rates the experimental biomass values were still markedly lower than predicted by the Monod model (Fig. 4), probably as a consequence of substrate carbon channelled to acetic acid production.

Similar biomass and LKT concentrations were obtained when the cysteine component of the cysteine plus glutamine mixture was replaced with sodium sulphide (Table 1), indicating that cultures grown in SDM1 became partly sulphur-limited at high dilution rates. It has been

suggested that *M. haemolytica* did not readily reduce sulphate for incorporation into sulphur-containing amino acids [11], which may explain the requirement for cysteine.

Conclusions

This study showed that the apparent growth factor limitation in cultures of *M. haemolytica* strain OVI-1 grown at high dilution rates in carbon-limited chemostat culture could be abolished by supplementation with a high concentration of yeast extract, but this failed to improve LKT production. The LKT concentration and yield on biomass ($Y_{LKT/x}$) could substantially be enhanced by supplementation with amino acid mixture B or a mixture of cysteine and glutamine, with or without vitamins and trace elements. LKT production was partially growth-associated. A large proportion of glucose carbon was chanelled to acetic acid production, especially under carbon-sufficient conditions, to the detriment of biomass and LKT production at high dilution rates. Acetic acid production could be minimised and the LKT concentration and $Y_{LKT/x}$ value maximised by operating a chemostat under a carbon limitation at a low dilution rate. This mode of continuous cultivation would also be applicable to fed-batch cultures. The use of low dilution rates would, however, suffer from the drawback of a low volumetric LKT productivity, but this could be remedied by using a high cell concentration.

Acknowledgment This work was supported by a grant from the National Research Foundation, South Africa.

References

1. Bell GH (1972) Yield factors and their significance. Process Biochem 7(4):21–24, 34
2. Carter BLA, Bull AT, Pirt SJ, Rowley BI (1971) Relationship between energy substrate utilization and specific growth rate in *Aspergillus nidulans*. J Bacteriol 108:309–313
3. Collier JR (1968) Significance of bacteria in bovine respiratory disease. J Am Vet Med Assoc 153:1645–1651
4. Confer AW, Clinkenbeard KD, Gatewood DM, Driskel BA, Montelongo M. (1997) Serum antibody responses of cattle vaccinated with partially purified native *Pasteurella haemolytica* leukotoxin. Vaccine 15:1423–1429
5. Conlon JA, Shewen PE, Lo RYC. (1991) Efficacy of recombinant leukotoxin in protection against pneumonic challenge with live *Pasteurella haemolytica* A1. Infect Immun 59:587–591
6. Djavan A, James AM (1980) Determination of the maintenance energy of *Klebsiella aerogenes* growing in continuous culture. Biotechnol Lett 2:303–308
7. Doelle HW, Hollywood N, Westwood AW (1974) Effect of glucose concentration on a number of enzymes involved in the aerobic and anaerobic utilization of glucose in turbidostat-cultures of *Escherichia coli*. Microbios 9:221–232
8. Gentry MJ, Confer AW, Panciera RJ (1985) Serum neutralization of cytotoxin from *Pasteurella haemolytica*, serotype 1 and resistance to experimental bovine pneumonic pasteurellosis. Vet Immunol Immunopathol 9:239–250
9. Gentry MJ, Confer AW, Weinberg ED, Homer JT (1986) Cytotoxin (leukotoxin) production by *Pasteurella haemolytica*: requirement for an iron-containing compound. Am J Vet Res 47:1919–1923
10. Han K, Lim HC, Hong J (1992) Acetic acid formation in *Escherichia coli* fermentation. Biotechnol Bioeng 39:663–671
11. Highlander SK (1997) Growth of *Pasteurella haemolytica* and production of its leukotoxin in semi-defined media. Am J Vet Res 58:749–754
12. Hollywood N, Doelle HW (1976) Effect of specific growth rate and glucose concentration on growth and glucose metabolism of *Escherichia coli* K-12. Microbios 17:23–33
13. Holms H (1996) Flux analysis and control of the central metabolic pathways in *Escherichia coli*. FEMS Microbiol Rev 19: 85–116
14. Karpinski KF, Hayward S, Tryphonas H (1987) Statistical considerations in the quantitation of serum immunoglobulin levels using the enzyme-linked immunosorbent assay (ELISA). J Immunol Meth 103:189–194
15. Kim T-B, Song S-H, Kang S-C, Oh D-K (2003) Quantitative comparison of lactose and glucose utilization in *Bifidobacterium longum* cultures. Biotechnol Prog 19:672–675
16. Kovárová K, Zehnder AJB, Egli T (1996) Temperature-dependent growth kinetics of *Escherichia coli* ML 30 in glucose-limited continuous culture. J Bacteriol 178:4530–4539
17. Kucera CJ, Wong JCS, Feldner TJ (1983) Challenge exposure of cattle vaccinated with a chemically altered strain of *Pasteurella haemolytica*. Am J Vet Res 44:1848–1852
18. Lendenmann U, Snozzi M, Egli T (2000) Growth kinetics of *Escherichia coli* with galactose and several other sugars in carbon-limited chemostat culture. Can J Microbiol 46:72–80
19. Majewski RA, Domach MM (1990) Simple constrained-optimization view of acetate overflow in *E. coli*. Biotechnol Bioeng 35:732–738
20. Mosier DA, Panciera RJ, Rogers DP, Uhlich GA, Butine MD, Confer AW, Basaraba RJ (1998) Comparison of serologic and protective responses induced by two *Pasteurella* vaccines. Can J Vet Res 62:178–182
21. Neijssel OM, Tempest DW (1976) Bioenergetic aspects of aerobic growth of *Klebsiella aerogenes* NCTC 418 in carbon-limited and carbon-sufficient chemostat culture. Arch Microbiol 107: 215–221
22. Nielsen J, Villadsen J (1994) Bioreaction engineering principles. Plenum, New York
23. Pirt SJ (1975) Principles of microbe and cell cultivation. Blackwell, Oxford
24. Sauer U, Hatzimanikatis V, Hohmann H-P, Manneberg M, van Loon APGM, Bailey JE (1996) Physiology and metabolic fluxes of wild-type and riboflavin-producing *Bacillus subtilis*. Appl Environ Microbiol 62:3687–3696
25. Senn H, Lendenmann U, Snozzi M, Hamer G, Egli T (1994) The growth of *Escherichia coli* in glucose-limited chemostat cultures: a re-examination of the kinetics. Biochim Biophys Acta 1201:424–436
26. Shewen PE, Wilkie BN (1985) Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. Am J Vet Res 46:1212–1214
27. Sreevatsan S, Ames TR, Werdin RE, Yoo HS, Maheswaran SK. (1996) Evaluation of three experimental subunit vaccines against pneumonic pasteurellosis in cattle. Vaccine 14:147–154
28. Srinand S, Ames TR, Werdin RE, Yoo SH, Maheswaran SK (1996) Evaluation of three experimental subunit vaccines against pneumonic pasteurellosis in cattle. Vaccine 14:147–154

29. Strathdee CA, Lo RYC. (1989) Regulation of expression of the *Pasteurella haemolytica* leukotoxin determinant. J Bacteriol 171:5955–5962
30. Sun Y, Clinkenbeard KD (1998) Serum-free culture of *Pasteurella haemolytica* optimized for leukotoxin production. Am J Vet Res 59:851–855
31. Tsai SP, Lee YH (1990) A model for energy-sufficient culture growth. Biotechnol Bioeng 35:138–145
32. Urban-Chmiel R, Wernicki A, Puchalski A, Mikucki P (2004) Evaluation of *Mannheimia haemolytica* leukotoxin prepared in nonsupplemented and BSA or FBS supplemented RPMI 1640 medium. Pol J Vet Sci 7:1–8
33. Van Rensburg E, du Preez JC, Ellis CE (2006) Quantification of *Mannheimia haemolytica* leukotoxin by indirect ELISA. Onderstepoort J Vet Res 73:241–250
34. Wessman GE (1965) Cultivation of *Pasteurella haemolytica* in a casein hydrolysate medium. Appl Microbiol 13:426–431
35. Wessman GE (1966) Cultivation of *Pasteurella haemolytica* in a chemically defined medium. Appl Microbiol 14:597–602