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Cheese whey: A cost-effective alternative for hyaluronic acid production by *Streptococcus zooepidemicus*

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ABSTRACT

This study focuses on the optimisation of cheese whey formulated media for the production of hyaluronic acid (HA) by *Streptococcus zooepidemicus*. Culture media containing whey (W; 2.1 g/L) or whey hydrolysate (WH; 2.4 g/L) gave the highest HA productions. Both W and WH produced high yields on protein consumed, suggesting cheese whey is a good nitrogen source for *S. zooepidemicus* production of HA. Polysaccharide concentrations of 4.0 g/L and 3.2 g/L were produced in W and WH in a further scale-up to 5 L bioreactors, confirming the suitability of the low-cost nitrogen source. Cheese whey culture media provided high molecular weight (>3000 kDa) HA products. This study revealed replacing the commercial peptone by the low-cost alternative could reduce HA production costs by up to a 70% compared to synthetic media.

1. Introduction

Hyaluronic acid (HA) is a linear polysaccharide composed of dimeric units of N-acetyl glucosamine and glucuronic acid. This polymer is a constituent of tissues such as skin, cartilage, umbilical cord, bird crests, synovial fluid, vitreous humour and it is also present in the cell walls of bacteria like *Streptococcus zooepidemicus* (Shiedlin et al., 2004). This gram-positive bacteria, a member of the group C streptococci, is facultative anaerobe, catalase-negative and has complex nutrient requirements on organic nitrogen, which supplies a large proportion of the carbon for cellular biosynthesis (Armstrong, Cooney, & Johns, 1997).

The biological function and specific application of HA depend on the molecular weight (Jagannath & Ramachandran, 2010). High molecular weight HA has an increasing level of demand in the clinical and pharmaceutical sectors, including plastic surgery, treatment of arthritis, major burns and intra-ocular surgery (Kogan, Šoltés, Stern, & Gemeiner, 2007). Also because it is biodegradable, highly biocompatible, contains reactive functional groups and can target specific cell surface receptors, HA is being widely studied as encapsulating material for the controlled release of therapeutic agents (Chen, Miller, & Dhal, 2014).

Conventionally HA was extracted from animal tissues (rooster combs, bovine vitreous humour and human umbilical cord) but now it is increasingly produced by fermentation of *S. zooepidemicus* (Vázquez et al., 2013). The increased attention on microbial HA is due to some advantages such as avoiding the risk of cross-species viral infection, a more efficient purification, lower production costs and higher yields compared to animal sources (Yamada & Kawasaki, 2005). However, the increasing price of culture media, mainly nitrogen and carbon sources (Vázquez, Montemayor, Fraguas, & Murado García, 2010), reduces the commercial competitiveness of this alternative. The formulation of cost-effective culture media is then a key topic to maintain the low costs of microbial HA production compared to the extraction from animal sources.

Recent papers have explored different agricultural resources and industrial wastes as alternative nutritive sources of microbial HA (Benedini & Santana, 2013; de Macedo & Santana, 2012; Pires, Macedo, Eguchi, & Santana, 2010; Vázquez, Montemayor, Fraguas, & Murado García, 2009; Vázquez et al., 2010). Cashew apple fruit bagasse was found to be an appropriate fermentation medium to produce low molecular weight HA in solid-state cultivation (de Macedo & Santana, 2012), due to its high content in B-vitamins and ascorbic acid. Marine peptones from fishing

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by-products were successfully used as substrates for the production of HA, biomass and lactic acid by *S. zooepidemicus* in submerged batch (Vázquez et al., 2010) and fed-batch (Vázquez et al. 2009) fermentations. On the other hand, Pires et al. (2010) reported the use of cheese whey protein concentrate as a nitrogen source for *S. zooepidemicus* cultivation in Erlenmeyer flasks but with low polysaccharide productions (0.17 g/L).

Whey is the main by-product of the cheese manufacturing industry (85–95% of the milk volume), consisting on the watery portion formed during the coagulation of milk casein (Guimaraes, Teixeira, & Domingues, 2010). The major nutrients in cheese whey are lactose (4.5–5% w/v), soluble proteins (0.6–0.8% w/v), lipids (0.4–0.5% w/v), mineral salts (8–10% of dried extract), lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins (Siso, 1996). *S. zooepidemicus* requires complex organic nitrogen but also has a limited ability to synthesize specific amino acids and B-vitamins (Armstrong et al., 1997). These nutritional requirements suggest cheese whey can be a good substrate for the production of HA by *S. zooepidemicus* due to its rich nutritional content. However, small-scale cultivation conditions do not enable a suitable pH, agitation and/or aeration control, being the production of HA extremely limited (Johns, Goh, & Oeggerli, 1994; Liu, Wang, Du, & Chen, 2008).

The aim of this work was to optimise the HA production by *S. zooepidemicus* using cheese whey as a low-cost nitrogen source. The production of HA in culture media formulated using cheese whey (W), concentrated cheese whey (WPC), and their hydrolysates (WH and WPH) was investigated in batch cultures. Cultivation of *S. zooepidemicus* in the culture media providing the best productions was further scaled-up in 5 L batch bioreactors. Cultivation performances and average molecular weight products were compared to those of synthetic medium.

2. Material and methods

2.1. Microorganisms

We utilised the HA-producing strain *Streptococcus equi* subsp. zooepidemicus ATCC 35246. Stock cultures were stored at −80 °C in complex medium (CM) with 25% glycerol (Vázquez et al., 2009). The inoculum was prepared following the methodology reported by Armstrong et al. (1997), as detailed in Vázquez et al. (2009).

2.2. Preparation of culture media

Four culture broths were formulated (Fig. 1) using cheese whey (W) or concentrated cheese whey (WPC). WPC was the concentrated fraction after 10 kDa ultrafiltration of W, and both provided by an Arzúa-Ulloa DOP cheese factory (Queizúar SL, A Coruña, Spain). The initial W composition was: 5.90 ± 0.61 g/L protein and 38.1 ± 0.82 g/L reducing sugars, of which 35.0 ± 1.17 g/L were lactose and 3.42 ± 0.41 g/L glucose. WPC had a protein content of 38.60 ± 2.29 g/L, 13.13 ± 0.51 g/L reducing sugars, of which 10.22 ± 1.84 g/L were lactose and 1.30 ± 0.25 g/L glucose.

Both W and WPC were hydrolysed using Alcalase 2.4 L from *Bacillus licheniformis* (Novozyme Nordisk, Bagsvaerd, Denmark). Hydrolysis was carried out with an enzyme/substrate ratio of 9.6 U/kg soluble protein, at 45 °C under orbital agitation (100 rpm) for 2 h. Then, samples were boiled for 15 min and cooled in an ice-water bath. The hydrolysates WH and WPH were centrifuged at 15,000×*g* for 20 min in an Avanti J-26XP centrifuge (Beckman Coulter, Inc., Miami, USA), and supernatants supplemented with sugars (glucose or lactose), yeast extract and salts at the same level of complex medium (CM).

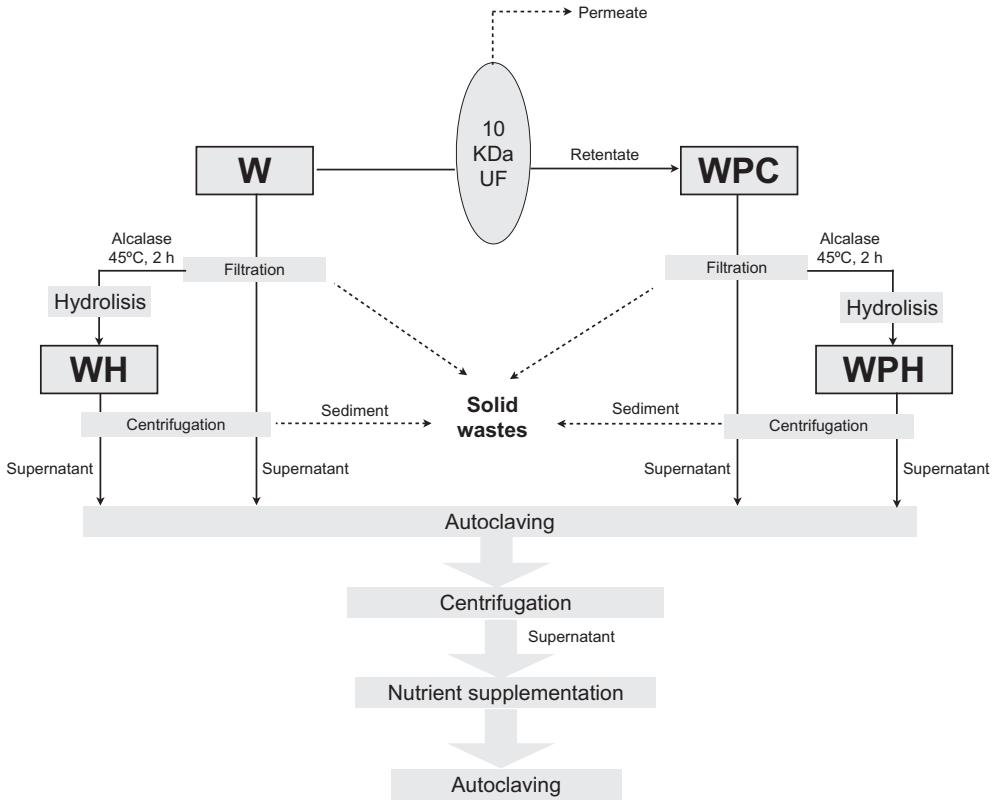

Fig. 1. Scheme illustrating culture media preparation.

Table 1 shows the composition of the four culture media prepared from W, WPC, WH and WPH. Yeast extract and tryptone were purchased from Cultimed (Panreac Química, Spain), glucose, lactose and salts were analytical grade and purchased from Sigma–Aldrich (St. Louis, MO). All culture media were sterilised at 121 °C for 15 min, and the initial pH was adjusted to 6.7.

2.3. Culture conditions

Cultures in W, WPC, WH and WPH were carried out in a 4-vessel glass 0.75 L-bioreactor with a working volume of 0.5 L (Biostat Q, Braun Sartorius). A 2-vessel 5 L-bioreactor with a working volume of 4.5 L (Biostat B, Braun Sartorius) was utilised to scale-up the cultures in W and WH media. All fermentations were at 37 °C, aeration of 1 vvm, agitation of 500 rpm, and the pH (6.7) automatically controlled with sterile 5 M NaOH.

2.4. Sampling and analytical methods

At pre-established times, samples were taken from the bioreactor and incubated with a 10% of 5% (w/v) SDS for 10 min. The biomass was removed by centrifugation at 15,000×*g* for 15 min and the optical density (OD) measured at 700 nm. The content of reducing sugars, soluble proteins, lactic acid, acetic acid, glucose (or lactose), and HA was determined in cell-free supernatants. The HA produced by *S. zooepidemicus* was selectively precipitated using ethanol (3:1), centrifuged at 10,000×*g* for 10 min, and redissolved in 1.5 M NaCl (1:1). Selective precipitation was repeated twice in the same conditions, the HA finally dissolved in distiled water, and the polymer concentration determined by the method reported by Blumenkrantz and Asboe-Hansen (1973) following the modifications proposed by Murado, Vázquez, Montemayor, Cabo, and González (2005).

Reducing sugars were analysed using the 3,5-dinitrosalicylic reaction (Bernfeld, 1951) and soluble proteins by the method of Lowry, Rosebrough, Farr, and Randall (1951). Lactic acid, acetic acid, glucose and lactose were analysed by HPLC using an ION-300 column (Transgenomic, USA) with 6 mM sulphuric acid as the mobile phase (flow = 0.4 mL/min) at 65 °C and a refractive index detector. The molecular weight (MW) of HA was determined by size-exclusion chromatography with an Ultrahydrogel linear column (Waters, USA) with 0.1 M $NaNO_3$ as the mobile phase (flow = 0.8 mL/min) and a refractive-index detector. Standards of polystyrene sulphonate (Sigma) with different molecular weights (32, 77, 150, 330, 990 and 2600 kDa) were used for calibration.

Table 1
Composition of the culture media utilised in the present work (g/L).

	W[a]	WH[b]	WPC[c]	WPH[d]	CM[e]
Glucose	50.00[f]	50.00[f]	–	–	50.00
Lactose	50.00[g]	50.00[g]	50.00[f]	50.00[f]	–
Yeast extract	5.00	5.00	5.00	5.00	5.00
Tryptone	–	–	–	–	15.00
KH_2PO_4	2.00	2.00	2.00	2.00	2.00
K_2HPO_4	0.50	0.50	0.50	0.50	0.50
$MgSO_4$	0.50	0.50	0.50	0.50	0.50
$(NH_4)_2SO_4$	0.50	0.50	0.50	0.50	0.50
Cheese whey protein (Lowry)	5.00	8.50	9.00	19.0	–

[a] W: cheese whey medium.
[b] WH: cheese whey hydrolysate medium.
[c] WPC: cheese whey protein concentrate medium.
[d] WPH: cheese whey protein hydrolysate medium.
[e] CM: complex medium (control).
[f] Batch cultures in 5 L-bioreactor were supplemented with glucose.
[g] Batch cultures in 0.5 L-bioreactor were supplemented with lactose.

2.5. Mathematical models

The profiles of *S. zooepidemicus* growth (X), hyaluronic acid (HA), lactic acid (L) and acetic acid (A) productions were modelled using the following logistic equation:

$$P = \frac{P_{max}}{(1 + \exp[2 + (4v_p/P_{max})(\lambda_p - t)])} \quad (1)$$

where P_{max} is the maximum biomass or product production (g/L), v_P is the maximum growth or production rate (g/L h), and λ_P is the lag phase of growth or metabolite production (h).

3. Results and discussion

3.1. Screening of cheese whey culture media

Cheese whey was tested as an alternative nitrogen source for AH production by *S. zooepidemicus*. To study which protein form was more accessible to the microorganism, we formulated four nutritive broths where tryptone was replaced by cheese whey (W), concentrated cheese whey (WPC), hydrolysed W (WH), or hydrolysed WPC (WPH) (Fig. 1). The reducing sugars content was adjusted with lactose, and all culture media supplemented with yeast extract and salts to the levels of the synthetic medium (CM; Table 1).

Fermentation performances were assessed according to biomass, metabolite production (hyaluronic, lactic and acetic acid) and substrate consumption (reducing sugars and protein) (Fig. 2 and Table 2). CM produced the best results of biomass production while WPC and WPH had the lowest (K) and slowest (v_X) biomass productions (Table 2) among the four cheese whey culture media. *S. zooepidemicus* grown in WH consumed 30 g/L lactose (Fig. 2h) leading to the highest biomass production (1.57 g/L; Fig. 2a) but in general, lactose uptake was low. Glucose showed a faster consumption than lactose, being totally depleted within 4–6 h in cheese whey cultures and after 8 h in CM (Fig. 2g, inset). In agreement with these findings, Chong and Nielsen (2003) reported a faster *S. zooepidemicus* growth on glucose ($\mu_{max} = 1.02\ h^{-1}$) than on maltose ($\mu_{max} = 0.84\ h^{-1}$), and Liu, Wang, Du, et al. (2008) found a high inhibition of *S. zooepidemicus* growth in culture media containing sucrose.

Synthetic medium (CM) reached the highest concentrations (3.37 and 37.9 g/L) and maximum production rates (0.73 and 8.34 g/L h) of HA and LA, respectively (Table 2). *S. zooepidemicus* produced 2.14 g/L HA in W, 2.38 g/L in WH, low concentrations (0.85 g/L) in WPH after 10 h of latency and no production at all in WPC. The higher productions compared to those previously reported in WPC (0.10–0.13 g/L) must be due to *S. zooepidemicus* cultivation under optimised conditions instead of erlenmeyer flasks (Pires et al., 2010) without pH control. It is known the production of lactic acid, the main by-product of HA fermentation (Liu, Liu, Li, Du, & Chen, 2011), lowers the pH and inhibits cell growth and HA synthesis (Liu, Wang, Sun, Du, & Chen, 2008). Besides, high agitation rates and aeration improves HA yields (Johns et al., 1994) and increases product molecular weights (Jagannath & Ramachandran, 2010).

The yields of LA and HA formation per biomass produced and per protein consumed were higher in W and WH than in the control (Table 2), supporting whey protein is an alternative nitrogen source for the production of HA by *S. zooepidemicus* under optimal cultivation conditions. Major proteins in cheese whey, ß-lactoglobulin, α-lactalbumin, immunoglobulins, serum albumin, and proteose peptones (de la Fuente, Hemar, Tamehana, Munro, & Singh, 2002), contain a higher proportion of essential amino acids and have a high protein efficiency ratio (3.4) compared to casein

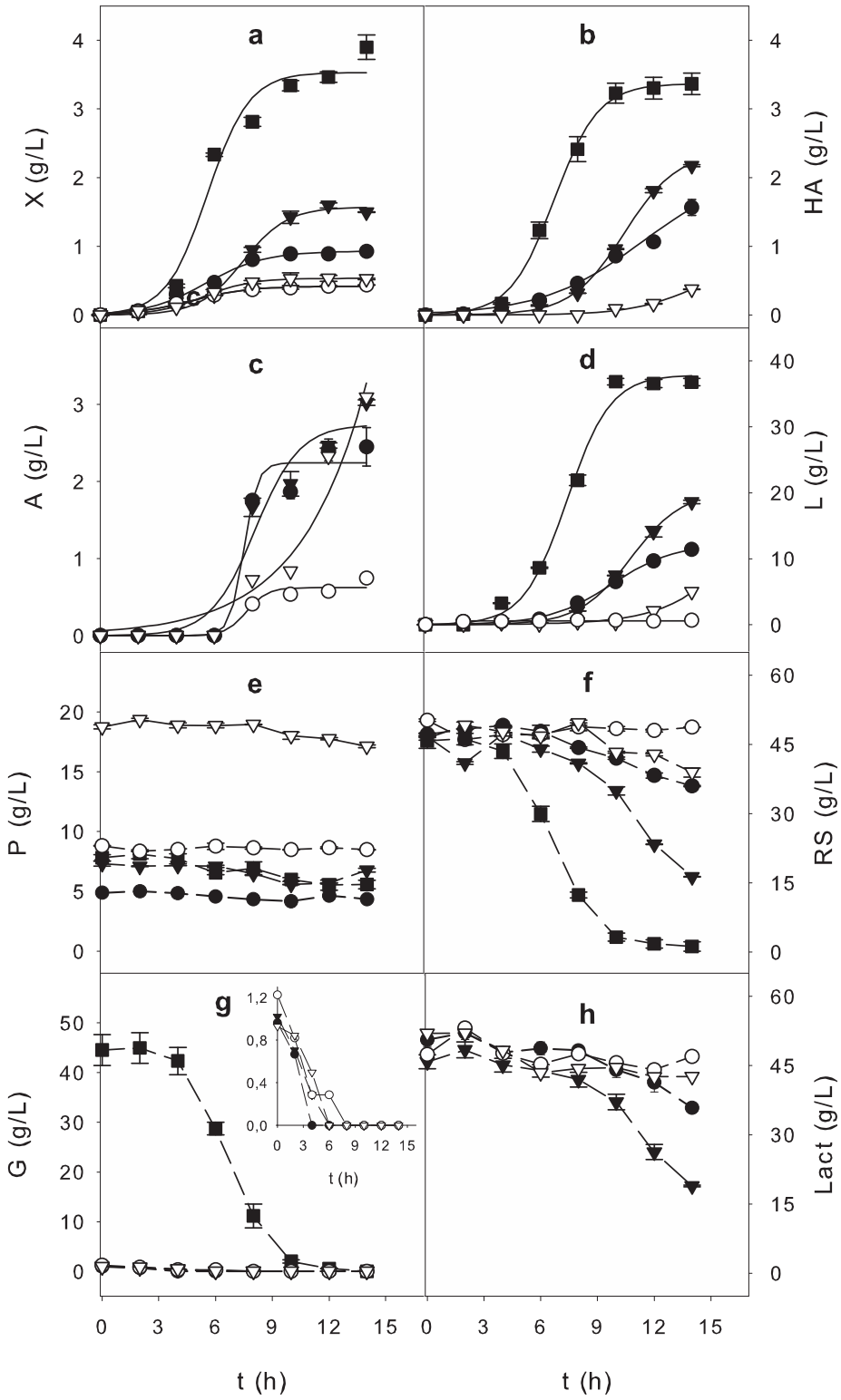

Fig. 2. Metabolic productions and substrate consumptions in small-scale batch cultures of *Streptococcus zooepidemicus* in cheese whey (W; ●), whey hydrolysate (WH; ▼), whey protein concentrate (WPC; ○), whey protein hydrolysate (WPH; ▽) and complex (CM; ■) media. Continuous lines are the fittings of the experimental results (points) according to Eq. (1). X: biomass (a); HA: hyaluronic acid (b), A: acetic acid (c), L: lactic acid (d), P: protein (e); RS: reducing sugars (f); G: glucose (g) and Lact: lactose (h). The graph inset is an enlarged representation of the glucose consumption.

(2.8) (Siso, 1996). Alcalase effectively hydrolyses cheese whey ß-lactoglobulin and α-lactalbumin (Kim et al., 2007), producing more complex peptide mixtures than tryptic casein digests, and being a potential alternative to commercial peptones for *S. zooepidemicus* growth. The lower yields observed in WPC and WPH media (Table 2) could be due to hindered accessibility of the microorganism to the thermal treated (autoclaving) protein. The formation of whey protein aggregates occurs as a consequence of heating or pressurising (Nicolai, Britten, & Schmitt, 2011). Moreover, whey protein concentrated solutions undergo faster thermal

Table 2
Parametric estimations corresponding to Eq. (1) applied to the production of biomass, HA, lactic and acetic acids by *Streptococcus zooepidemicus* in W, WPC, WH, WPH and CM. CI: confidence intervals ($\alpha = 0.05$). r^2 = correlation coefficient between observed and predicted data. NS: not significant.

	W	WPC	WH	WPH	CM
Biomass					
K (g/L)	0.93 ± 0.08	0.42 ± 0.02	1.57 ± 0.14	0.53 ± 0.03	3.53 ± 0.45
v_X (g/L h)	0.15 ± 0.05	0.07 ± 0.02	0.34 ± 0.12	0.11 ± 0.03	0.81 ± 0.52
λ_X (h)	2.50 ± 1.06	1.77 ± 0.77	5.21 ± 0.88	2.97 ± 0.66	3.46 ± 1.58
r^2	0.991	0.996	0.994	0.996	0.978
$Y_{X/RS}$ (g/g)	0.08	0.10	0.05	0.07	0.09
$Y_{X/P}$ (g/g)	1.75	1.35	2.72	0.32	1.74
Hyaluronic acid					
HA (g/L)	2.14 ± 1.14	ND	2.38 ± 0.19	0.85 (NS)	3.37 ± 0.15
v_{HA} (g/L h)	0.20 ± 0.05	ND	0.43 ± 0.05	0.12 (NS)	0.73 ± 0.13
λ_{HA} (h)	6.07 ± 1.50	ND	7.66 0.35	10.9 ± 3.78	4.47 ± 0.48
r^2	0.991	–	0.999	0.991	0.998
$Y_{HA/X}$ (g/g)	1.69	–	1.45	0.71	0.86
$Y_{HA/RS}$ (g/g)	0.14	–	0.07	0.04	0.08
$Y_{HA/P}$ (g/g)	2.96	–	3.95	0.23	1.50
Lactic acid					
LA (g/L)	12.3 ± 1.04	0.60 ± 0.10	20.3 ± 1.37	7.25 (NS)	37.9 ± 3.16
v_{LA} (g/L h)	1.84 ± 0.23	0.62 (NS)	3.74 ± 0.36	1.56 (NS)	8.34 ± 2.76
λ_{LA} (h)	6.47 ± 0.43	1.25 (NS)	8.10 ± 0.27	10.7 (NS)	5.19 ± 0.84
r^2	0.998	0.885	0.999	0.993	0.994
$Y_{LA/X}$ (g/g)	12.3	1.53	12.4	9.56	9.38
$Y_{LA/RS}$ (g/g)	1.02	0.16	0.61	0.49	0.82
$Y_{LA/P}$ (g/g)	21.6	2.06	33.8	3.08	16.3
Acetic acid					
A (g/L)	2.24 ± 1.04	0.62 ± 0.11	2.73 ± 0.71	3.81 ± 1.95	ND
v_{AA} (g/L h)	1.55 ± 0.23	0.28 (NS)	0.59 ± 0.54	0.58 ± 0.22	ND
λ_{AA} (h)	6.82 ± 0.43	6.59 ± 2.56	5.81 ± 2.34	8.22 ± 1.29	ND
r^2	0.986	0.965	0.957	0.980	–
$Y_{AA/X}$ (g/g)	2.64	1.71	2.02	5.90	–
$Y_{AA/RS}$ (g/g)	0.22	0.18	0.10	0.30	–
$Y_{AA/P}$ (g/g)	4.62	2.30	5.49	1.90	–

denaturation processes (Wolz & Kulozik, 2015), which would explain the lower yields found in WPC and WPH compared to W and WH.

The average molecular weight of HA produced in W (3764 ± 38 kDa) and WH (3474 ± 114 kDa) media was higher than in CM (3105 ± 93 kDa). Previous findings have shown the use of different carbon sources alters the glycolytic end products (Thomas, Turner, & Crow, 1980), and regulates the molecular weight of HA (Jagannath & Ramachandran, 2010). The use of complex sugars such as starch (Zhang, Ding, Yang, & Kong, 2006) and glycogen (Vázquez et al., 2010) produced reduced rates of lactate production, shuttling the carbon flux towards HA synthesis and leading to higher molecular weight products. In agreement with the present results, other reports have found lower lactic acid production linked to high HA molecular weight products in by-product formulated media (Benedini & Santana, 2013; Liu, Wang, Sun, et al., 2008; Vázquez et al., 2010).

3.2. Scale-up of batch cultures in whey (W) and whey hydrolysate (WH)

A 10-fold scale-up in 5 L bioreactors was carried out in W and WH (Fig. 3 and Table 3), using glucose instead of lactose as the carbon source (Table 1) due to the rather low disaccharide consumption observed in 0.5 L bioreactor cultures (Fig. 2h).

After a lag phase of 3.4 h, *S. zooepidemicus* produced a maximum biomass concentration of 6.02 g/L in W medium while, after 5 h, the cells entered the exponential growth phase (Fig. 3) with a maximum biomass concentration of 4.39 g/L in CM medium (Table 3). On the other hand, maximum production rates were slightly higher (0.87 g/L h) and lower (0.53 g/L h) in W and WH respectively, compared to the control (0.75 g/L h). Biomass productions in W and WH were higher in 5 L (Table 3) than in 0.5 L bioreactors (Table 2), supporting glucose is preferred to lactose for biomass production. Cultivation in cheese whey media yielded similar metabolite (HA, LA and AA) productions to the control (Table 3), but a slightly higher HA production was observed in W (4.02 g/L) compared to WH and CM (3.19 g/L) media. The fact that glucose was utilised preferably for cell growth than HA synthesis, especially in W and WH, is in agreement with previous findings (Chong, Blank, Mclaughlin, & Nielsen, 2005; Liu, Wang, Du, et al., 2008).

Slightly higher biomass and product (HA and LA) formation were found compared to media formulated using marine peptones (Vázquez et al., 2009; Vázquez et al., 2010) as protein sources, under similar conditions as those of the present research (temperature, pH, agitation). These results suggest whey protein could be a better alternative to marine peptones, despite the excellent viability of fish by-product formulated media for HA production by *S. zooepidemicus*.

The yields of HA, LA and AA production to nutrient uptake, particularly protein ($Y_{HA/P}$, $Y_{LA/P}$ and $Y_{AA/P}$), were more favourable in cheese whey culture media, which confirms our previous findings on small-scale cultivation. The HA average molecular weights in CM, W and WH, were 2542 ± 213, 3714 ± 91 and 3321 ± 221 kDa respectively.

We estimated the costs (€) of CM, W and WH culture media based on the prices of commercial peptones, sugars, and rest of ingredients according to the compositions shown in Table 1. We assumed whey had no cost because currently, this pollutant residual effluent has to be treated by an authorised manager and implies a high cost for cheese producers. We also calculated

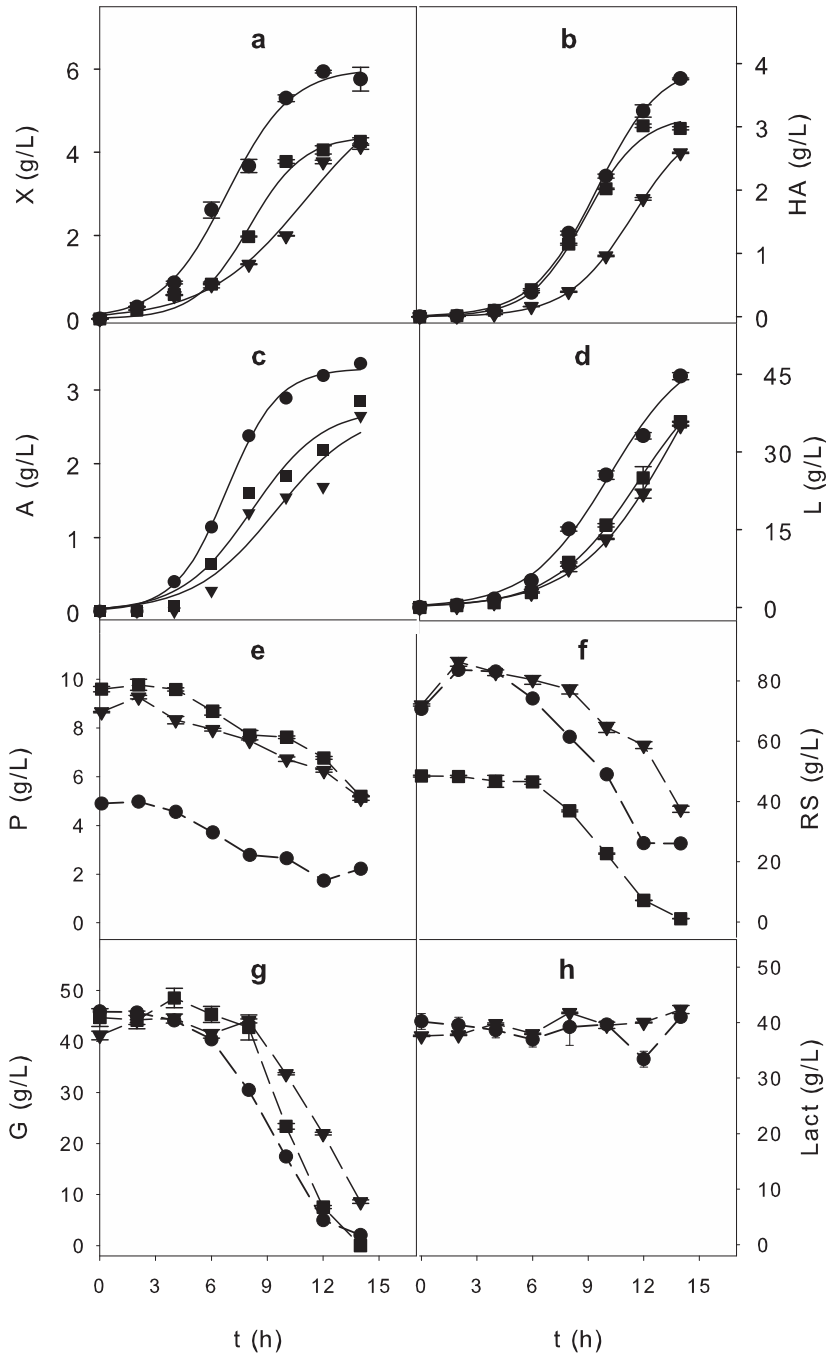

Fig. 3. Metabolic productions and substrate consumption in 5 L batch cultures of *Streptococcus zooepidemicus* in cheese whey (W; ●), whey hydrolysate (WH; ▼) and complex (CM; ■) media. Continuous lines are the fittings of the experimental results (points) according to Eq. (1). X: biomass (a); HA: hyaluronic acid (b), A: acetic acid (c), L: lactic acid (d), P: protein (e); RS: reducing sugars (f); G: glucose (g) and Lact: lactose (h).

the total amount of HA produced (g) in each medium, considering the maximal productions (Table 3), and the final culture volumes. This simple analysis of HA production costs (€/g) revealed a 72% and 65% fewer production costs in W and WH respectively (Fig. 4), compared to CM medium. This result highlights the importance of replacing the commercial nitrogen source by a low-cost alternative for an economically competitive HA production. However, at the same time, our approach is a promising strategy towards the valorization of cheese whey, by producing high value-added products such as hyaluronic and lactic acid.

4. Conclusions

In this study, we developed a low-cost culture medium using cheese whey as a nitrogen source for HA production by *S. zooepidemicus*. Culture media containing cheese whey (4.0 g/L) or whey hydrolysate (3.2 g/L) produced HA concentrations comparable to the synthetic medium (3.2 g/L), confirming the suitability of this alternative nitrogen source for this bioproduction. A simple cost analysis revealed the viability of the by-product formulated media to reduce the production costs by up to a 70%, compared to synthetic media.

Table 3
Parametric estimations corresponding to Eq. (1) applied to the production of biomass, HA, lactic and acetic acids by *Streptococcus zooepidemicus* in W, WH and CM. CI: confidence intervals ($\alpha = 0.05$). r^2 = correlation coefficient between observed and predicted data. NS: not significant.

	W	WH	CM
Biomass			
K (g/L)	6.02 ± 0.60	5.69 ± 3.44	4.39 ± 0.58
v_X (g/L h)	0.87 ± 0.24	0.53 ± 0.15	0.75 ± 0.28
λ_X (h)	3.37 ± 1.02	5.69 ± 1.77	5.10 ± 0.45
r^2	0.993	0.980	0.990
$Y_{X/RS}$ (g/g)	0.13	0.12	0.09
$Y_{X/P}$ (g/g)	2.15	1.17	0.97
Hyaluronic acid			
HA (g/L)	4.02 ± 0.39	3.19 ± 0.15	3.19 ± 0.37
v_{HA} (g/L h)	0.58 ± 0.09	0.46 ± 0.02	0.53 ± 0.14
λ_{HA} (h)	6.07 ± 0.21	7.94 ± 0.12	5.94 ± 0.81
r^2	0.998	0.999	0.995
$Y_{HA/X}$ (g/g)	0.65	0.63	0.70
$Y_{HA/RS}$ (g/g)	0.08	0.07	0.06
$Y_{HA/P}$ (g/g)	1.40	0.73	0.68
Lactic acid			
LA (g/L)	50.8 ± 12.7	67.3 ± 33.9	48.9 ± 11.3
v_{LA} (g/L h)	5.90 ± 0.48	6.42 ± 1.88	5.35 ± 0.55
λ_{LA} (h)	5.87 ± 0.93	8.56 ± 1.44	7.20 ± 0.56
r^2	0.993	0.998	0.998
$Y_{LA/X}$ (g/g)	7.75	8.47	8.41
$Y_{LA/RS}$ (g/g)	1.00	1.01	0.76
$Y_{LA/P}$ (g/g)	16.7	9.90	8.14
Acetic acid			
AA (g/L)	3.29 ± 0.17	2.74 ± 1.81	2.75 ± 0.81
v_{AA} (g/L h)	0.60 ± 0.11	0.31 ± 0.20	0.36 ± 0.20
λ_A (h)	4.08 ± 0.54	5.04 ± 3.06	4.38 ± 2.18
r^2	0.998	0.939	0.970
$Y_{AA/X}$ (g/g)	0.58	0.64	0.67
$Y_{AA/RS}$ (g/g)	0.08	0.08	0.06
$Y_{AA/P}$ (g/g)	1.25	0.75	0.65

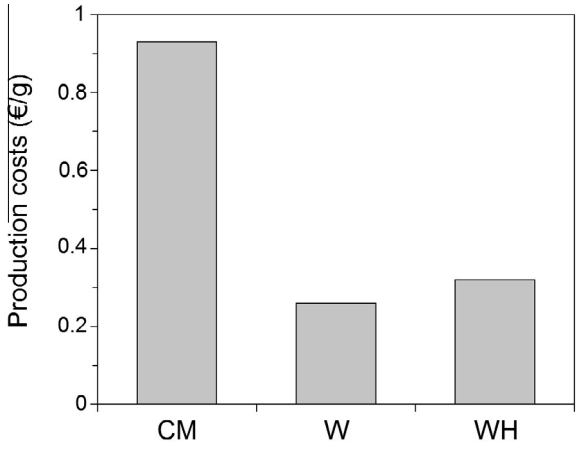

Fig. 4. HA production costs (€/g) in CM, W and WH in 5 L batch bioreactor.

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