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Microbial production of hyaluronic acid from agro-industrial by-products: Molasses and corn steep liquor

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ABSTRACT

Agro-industrial by-products are being explored as alternative low-cost nutrients to produce hyaluronic acid (HA) by *Streptococcus zooepidemicus*. In this study, we formulated three culture media containing corn steep liquor (CSL) and sugarcane molasses (M), to produce microbial HA using batch bioreactor conditions (pH 6.7, 500 rpm and 1 vvm aeration). Final HA concentrations of 3.48 g L^{-1} were produced in culture medium containing corn steep liquor (10% v/v) and glucose, being comparable (3.60 g L^{-1}) to the control medium containing tryptone and glucose. The use of molasses (10% v/v) as carbon source produced a marked inhibition of *S. zooepidemicus* growth and HA production due to a low sugar consumption. The HA produced in CSL culture media had a high molecular weight of 3.8×10^3 kDa, greater than HA produced in tryptone-containing medium (3.0×10^3 kDa).

This is the first report achieving HA productions comparable to synthetic a medium in a batch bioreactor using CSL as the main nitrogen source. However, further optimization of culture conditions must be carried out towards using this agricultural by-product for the sustainable industrial production of HA.

1. Introduction

Hyaluronic acid (HA) is a glycosaminoglycan found in vertebrate tissues as an essential component of the extracellular matrix. This polysaccharide has a linear structure consisting of β-1,3-*N*-acetyl glucosamine-β-1,4-Glucuronic acid disaccharide repeating units [1]. Despite the simplicity of its structure, the polymer is semi-flexible and adopts an expanded wormlike random coil conformation in solution [2], exhibiting an unusual rheological behaviour. HA is an attractive molecule for specific applications in the cosmetic [3], pharmacological [4] and medical sectors [5] due to its viscoelasticity together with other advantages such as biocompatibility, angiogenic and immunostimulatory properties.

HA was traditionally recovered from rooster combs, synovial fluid, vitreous humour and umbilical cords [6] from terrestrial animals, but also from marine supplies [7]. In recent years, the microbial production by *Streptococci* was extensively investigated [8] due to improved HA yields, more efficient downstream processes, and reduced risk of cross-species viral infection [9]. In spite of these advantages, microbial cultivation must be cost competitive with HA recovery from animal sources. *Streptococci* have complex nutrient requirements on organic nitrogen [10], and nutritive media commonly used to grow these microorganisms contain high amounts of rich nutrients [11]. The continuous increment in the cost of these raw materials reduces the commercial competitiveness of microbial HA production [8] and therefore, the use of low-cost renewable resources and agro-industrial by-products as culture media contributes towards making HA production economically feasible.

Molasses is a by-product of the sugar cane industry containing valuable compounds for the fermentation process like sucrose, minerals, organic compounds and vitamins [12]. CSL is a by-product of the corn wet milling industry rich in vitamins, minerals, amino acids and proteins, and an important source of nitrogen [13]. The high nutritive value of both substrates suggests they could be useful for the formulation of culture medium to produce HA using a bacterium with complex nutrient requirements like *S. zooepidemicus*.

The production of HA using renewable resources as ingredients for the formulation of culture media is being explored nowadays [14]. The substitution of commercial peptones by marine

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by-products [15–17], and cheese-whey protein [18] yielded high concentrations of HA in batch cultures of *Streptococcus zooepidemicus*. Vegetable by-products are also extensively explored because HA for cosmetic and pharmacological applications must be produced from non-animal sources [19]. De Macedo and Santana [20] found juice-moisturized bagasse with cashew apple fruit was a promising (>6 mg/g) source for the production of low molecular weight HA (10^4–10^5 Da) in solid-state fermentation. In another study, HA was produced in culture medium containing corn steep liquor (CSL, 0.86 g L^{-1}), or soy protein hydrolysate alone and combined with CSL (0.17 g L^{-1}) as nitrogen sources [21]. Pan et al. [22] reported the replacement of yeast extract by soy protein resulted in a polymer production of 0.22 g L^{-1} while these authors did not find any HA production using CSL as the nitrogen source.

The objective of this work is the development of a low-cost alternative medium for the production of HA by *S. zooepidemicus* using molasses and corn steep liquor. The appropriate conditions (pH control, agitation, and aeration) for the production of HA were first defined in culture medium containing glucose and tryptone, and performances compared to alternative culture media.

2. Material and methods

2.1. Strain and culture conditions

The HA-producing strain *Streptococcus equi* subsp. zooepidemicus ATCC 35246 was stored at −80 °C in complex medium (CM) with 25% glycerol. The composition of CM medium was (g L^{-1}): glucose 50.0; tryptone 15.0; yeast extract 2.75; KH_2PO_4 2.00; K_2HPO_4 2.0; $MgSO_4$ 2.0; $(NH_4)_2SO_4$ 0.5; pH 6.7. The inoculum consisted of a 10% (v/v) as reported by Armstrong et al. [10], and detailed in Vázquez et al. [15].

Cultures were carried out in 0.75 L-bioreactor with a working volume of 0.5 L (Biostat Q, Braun Sartorius), at 37 °C. We tested different agitation (200, 500 and 800 rpm) and aeration (0, 1 vvm) conditions in CM medium, and the pH maintained at 6.7 using 5 M NaOH.

2.2. Streptococcus zooepidemicus *culture using alternative substrates*

Sugarcane molasses, kindly provided by RAR: Refinarias de Açúcar Reunidas, S.A. (Portugal), and CSL kindly provided by COPAM: Companhia Portuguesa de Amidos, S.A. (Portugal) were the alternative substrates for HA production by *Streptococcus zooepidemicus* ATCC 35246. Molasses contained 495 g L^{-1} of carbohydrates and 20 g L^{-1} of protein, while CSL contained 69 g L^{-1} of carbohydrates, and 57 g L^{-1} of protein. Molasses (M) were dissolved in distilled water (10% (v/v)), and tested as culture media containing either tryptone or 10% (v/v) corn steep liquor (CSLM). A third culture medium containing 50 g L^{-1} glucose, and 10% (v/v) corn steep liquor was prepared (CSL). All media were supplemented with yeast extract, KH_2PO_4, K_2HPO_4, $MgSO_4$, and $(NH_4)_2SO_4$ at the same levels as CM medium (Table 1). The initial pH was adjusted to pH 6.7 and the cultures carried out under previously defined conditions (37 °C, 500 rpm, 1 vvm).

Table 1
Composition of culture media (g L^{-1}) utilised for the production of hyaluronic acid by *Streptococcus zooepidemicus* ATCC 35246. M: culture medium containing sugarcane molasses, CSL: culture medium containing corn steep liquor, CSLM: culture medium containing sugarcane molasses and corn steep liquor, CM: complex medium.

	M	CSL	CSLM	CM
Glucose	–	50.00	–	50.00
Yeast extract	5.00	5.00	5.00	5.00
Tryptone	15.0	–	–	15.00
KH_2PO_4	2.00	2.00	2.00	2.00
K_2HPO_4	0.50	0.50	0.50	0.50
$MgSO_4$	0.50	0.50	0.50	0.50
$(NH_4)_2SO_4$	0.50	0.50	0.50	0.50
Molasess (%v/v)	10.0	–	10.0	–
CSL (%v/v)	–	10.0	10.0	–

2.3. Analytical methods

Samples were taken at different time points of fermentation and incubated with a 10% of 5% (w/v) SDS for 10 min. Biomass was removed by centrifugation at 15000 × *g* for 15 min and the optical density (OD) measured at 700 nm. We quantified the concentration of total sugars and soluble proteins in the supernatant using the methods phenol-sulphuric [23] and Lowry [24], respectively. Glucose and sucrose were quantified 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 production of HA was quantified after selective precipitation in the supernatant using ethanol (3:1) followed by centrifugation (10000 × *g*, 10 min). The sediment was dissolved in 1.5 M NaCl (1:1) and re-precipitated under the same conditions. Finally, the HA was suspended in distilled water and the concentration determined by the method of Blumenkrantz and Asboe-Hansen [25], following the modifications proposed by Murado et al. [26]. 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.

2.4. Numerical and statistical analysis

S. zooepidemicus growth (X) kinetics were modelled using the following logistic equation [16]:

$$X = \frac{X_m}{\left(1 + exp\left[2 + \left(\frac{4\upsilon_X}{X_m}\right)(\lambda_X - t)\right]\right)} \quad (1)$$

where X is the biomass production (g L^{-1}), X_m is the maximum biomass (g L^{-1}), ν_x is the maximum growth rate (g L^{-1} h^{-1}) and λ_x is the growth lag phase (h).

Also a logistic equation was employed to model HA production data [16]:

$$H = \frac{H_m}{\left(1 + exp\left[2 + \left(\frac{4\upsilon_H}{H_m}\right)(\lambda_H - t)\right]\right)} \quad (2)$$

where H is the HA production (g L^{-1}), H_m is the maximum HA concentration (g L^{-1}), ν_H is the maximum HA production rate (g L^{-1} h^{-1}) and λ_H is the delay in HA production (h).

We calculated the yield of HA production on biomass by means of the following equation [27]:

$$H = Y_{H/x}\frac{X_m}{1 + exp\left[2 + \frac{4\upsilon_X}{X_m}(\lambda_X - t)\right]} - Y_{H/x}X_0 \quad (3)$$

where $Y_{H/x}$ is the yield of HA production per biomass (g HA g^{-1} biomass).

Plotting and data fitting were performed using the software GraphPad PrismTM 5 (GraphPad Software Inc., San Diego, CA, USA). The significance of the mathematical models (Fisher's F-test) was assessed using the "SolverAid" macro (Levie's Excellaneous website: http://www.bowdoin.edu/~rdelevie/excellaneous).