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Short Communication

Effects of soy peptone on the inoculum preparation of *Streptococcus zooepidemicus* for production of hyaluronic acid

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ABSTRACT

Soy peptone (SP) was studied as nutrient source in replacement of the conventional media as Brain–Heart Infusion (BHI) and sheep blood in the first seed culture medium in Petri plates of *Streptococcus zooepidemicus*. This substitution, aimed at meeting the claim of the pharmaceutical and cosmetics industries, for the removal of animal sources of the culture media used in obtaining their products for safety reasons. The animal sources were used as a control. The effects of this substitution were studied in fermentations carried out at 37 °C and 150 rpm in 250 mL Erlenmeyer flasks containing 100 mL culture medium containing glucose and SP only. The replacement of animal nutrient sources by SP to about twice the BHI concentration did not alter the amount of the produced HA, or caused deviations in the metabolism of the microorganism in favor of HA to the detriment of cell growth.

1. Introduction

Hyaluronic acid (HA) is a natural polysaccharide with a wide range of pharmaceutical, medical and cosmetic applications (Weigel, 2004). Due to its biological applications, it is necessary to produce it highly pure and free from viral agents. Nowadays, the microbial production is the preferred alternative, compared to the extraction from animal tissues such as rooster combs, umbilical cord blood or synovial fluid. The microbial route uses *Streptococci* mainly, which produce HA as a protective capsule around its cell wall (Chong et al., 2005; Weigel, 2004).

In general, the first seed culture media for propagation of *Streptococci* contain animal-derived sources, such as sheep blood and/or Brain Heart Infusion (BHI), for the cell growth (Pires et al., 2010a,b; Pires and Santana, 2010).

Although the animal sources are effective for cultivation of the microorganism, there is a risk of species cross contamination in HA produced, even when these components are used in the first steps of inoculum preparation, such as in Petri dishes growth.

Recently, vegetable peptones have been used as nitrogen sources in culture media for cell cultivation (Fakhfakh-Zouari et al., 2010; Heenan et al., 2002; Junguo et al., 2004; Le Marrec et al., 2007; Siaterlis et al., 2008), including the cultivation of *Streptococcus* and *HA production* (Cazzola et al., 1994; Izawa et al., 2010; Kanchankumar et al., 2009; Lee et al., 2009; Zisu and Shah, 2003). On the other hand, the pharmaceutical and cosmetics applications have demanded non-animal nutrients in microbial culture media for the production of their components, for safety reasons. As far as we know, there is no report in the literature about the use of vegetable peptones as a nitrogen source for growth of *Streptococcus zooepidemicus* and for the production of hyaluronic acid.

In this context, the objective of this study was to substitute BHI and sheep blood for soy peptone (SP) in the culture medium for the growth of *S. zooepidemicus* in Petri dishes. Soy protein was chosen because it is commercially available at low cost. The effects of the substitution were studied in fermentations, in which cell growth, HA production, glucose consumption, and secondary metabolites were quantified. Additionally, the average molecular weight and polydispersity of the produced HA was also characterized.

2. Methods

2.1. Microorganism

Streptococcus equi, subsp. *zooepidemicus* ATCC 39920, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) as a lyophilized culture kept frozen at −80 °C in ampoules containing 10% glycerol as cryoprotectant.

2.2. Solid inocula preparations

In the first culture in Petri dishes, the compositions of the solid medium were: P_c or control (37 g L^{-1} BHI, 5% (v/v) sheep blood); P_I

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(67 g L^{-1} SP, 5% (v/v) sheep blood) and P_{II} (67 g L^{-1} SP) were the compositions of the solid medium with sequential replacement of BHI and sheep blood. The SP was used at 67 g L^{-1} concentration, according to preliminary studies (data not shown). All the experiments were performed in triplicate.

2.3. Culture medium for liquid inocula

The culture medium for liquid inocula and fermentations was composed by 33.5 g L^{-1} SP and 25 g L^{-1} glucose, according to the 7.8 glucose/nitrogen ratio (Pires et al., 2010a,b; Pires and Santana, 2010).

2.4. Fermentations

Three fermentations were performed from P_c, P_I and P_{II} inocula. They were carried out in 250 mL Erlenmeyer flasks containing 100 mL culture medium and 10% (v/v) for each inoculum. The flasks were maintained under reciprocal shaking, at 37 °C, 150 rpm during 24 h. Cell mass, HA production, HA molecular weight, glucose consumption and the concentrations of acetic, lactic and formic acid secondary metabolite were determined at the initial and final times. The analyses were performed in triplicate.

2.5. Analytical methods

2.5.1. Cell growth

Cell growth was determined by cell dry weight according to the gravimetric method. To this end, 10 mL of the culture broth were centrifuged in tubes previously dried to constant weight at 60 °C. After centrifugation, the cell pellet was washed twice with deionized water and dried to constant weight under these same conditions.

2.5.2. Glucose concentration

Glucose concentration was determined using a glucose oxidase commercial kit (LABORLAB Ltda, Guarulhos, São Paulo, Brazil).

2.5.3. HA concentration

Initially the fermented medium was centrifuged at 22,400*g* for 20 min. The cell free supernatant was treated with ethanol in a proportion 1.5:1 (v/v) ethanol:supernatant. The solution was then cooled down, remaining at 4 °C during 1 h for the precipitation of HA. The HA precipitated was redissolved in a 0.15 mol L^{-1} NaCl solution. Three steps of precipitation and redissolution were performed to increase the purity of HA precipitated. The concentration of HA was determined by the carbazol reagent (Dische, 1946). Sodium hyaluronate (Hylumed™) from Genzyme Corporation (Cambridge, MA, USA) was used as a standard.

2.5.4. Concentrations of lactate, acetate and, formate

Culture samples were filtered through membranes with a pore size of 0.2 μm (Sartorius, Goettingen, Germany), and 20 μL of filtered sample was injected into an ion exchange High Performance Liquid Chromatography (Shimadzu Corporation, Kyoto, Japan) equipped with a 7.8 × 300 mm HPX-87H fast acid column Aminex (Bio-Rad, Hercules, CA, USA). The mobile phase was composed of 0.004 mol L^{-1} H_2SO_4 solution pumped at a flow rate of 0.6 mL min^{-1} as described by Chong et al., 2005. The column was maintained at 65 °C. The peak elution profile was monitored with a Shimadzu RID-6A refractive index detector (Shimadzu Corporation, Kyoto, Japan).

2.5.5. HA molecular weight

The average molecular weight of HA was determined by size exclusion chromatography, using a Shimadzu chromatography system (Shimadzu Corporation, Kyoto, Japan), containing a 7.8 × 35 mm Polysep-GFC-P column guard (Phenomenex, Torrance, CA., USA) mounted in series with a 7.8 × 300 mm Polysep-GFC-P6000 column of the gel filtration (Phenomenex, Torrance, CA., USA) and a refraction index detector. The analysis conditions were: injected sample of 20 μL, 0.1 mol L^{-1} sodium nitrate as the mobile phase, 1.0 mL.min^{-1} flow rate and 25 °C temperature, as suggested by the column manufacturer. Dextran (American Polymer Standards, Mentor, OH, USA) with molecular weight ranging from 103 to 106 Da was used as a standard for the calibration curve as described by Balke et al. (1969).

2.5.6. Statistical analysis

All the data of this work were compared by the Tukey test at 5% probability level ($p < 0.05$).

3. Results and discussion

3.1. Performance of the inoculum from soy peptone

Table 1 shows the performance of the fermentations with the studied inocula compared to control.

The results from the gradual replacement of the animal nutrient sources by the vegetal source SP (Table 1) showed that the performance of the fermentations with replacement of BHI but maintenance of the sheep blood in the media was similar to the control in all parameters, except for the HA average molecular-weight. However, when BHI and sheep blood were substituted by SP in the solid medium, all the parameters were different from control, except for the HA production and the yield of HA related to glucose. Despite the statistical differences, there was no metabolic deviations in the fermentations in favor of HA at the expenses of cells ($Y_{P/X} < 1$) for none of the studied inocula. The increment of the HA molecular-weight in fermentations with the partial or total substitution of BHI and sheep blood, were directly related to the decreasing production of the lactic and acetic acids and preservation of HA exposition to a lower pH. The polydispersity of HA increased slightly when SP was used. Therefore, the solid medium of the first seed did not interfere on the performance of the inocula in the fermentations with SP. In general, the culture media used for

Table 1
Performance of the fermentations with substitutions of the animal nutrient sources, Brain Heart Infusion and sheep blood, by soy peptone in the first seed culture medium *Streptococcus zooepidemicus* (ATCC 39920). P_c or control is the conventional medium for propagation of *S. zooepidemicus* (37 g L^{-1} BHI, 5% (v/v) sheep blood); P_I (67 g L^{-1} SP, 5% (v/v) sheep blood) and P_{II} (67 g L^{-1} SP) were the compositions of the solid medium with sequential replacement of BHI and sheep blood.

Parameter	P_C	P_I	P_{II}
X_f (g L^{-1})	0.31 ± 0.00[a]	0.42 ± 0.06[b]	0.30 ± 0.04[a]
Cell growth (g L^{-1})	1.85 ± 0.17[a]	2.00 ± 0.22[a]	2.63 ± 0.26[b]
Glucose consumption (g L^{-1})	7.25 ± 0.74[a]	7.29 ± 0.51[a]	8.01 ± 0.64[b]
HA production (g L^{-1})	0.29 ± 0.03[a]	0.27 ± 0.03[a]	0.30 ± 0.04[a]
$Y_{P/S}$	0.04 ± 0.01[a]	0.04 ± 0.01[a]	0.04 ± 0.01[a]
$Y_{P/X}$	0.16 ± 0.03[a]	0.14 ± 0.02[a]	0.11 ± 0.02[b]
$Y_{X/S}$	0.25 ± 0.08[a]	0.29 ± 0.08[a]	0.36 ± 0.11[b]
Lactate (g L^{-1})	3.17 ± 0.21[a]	3.08 ± 0.32[a]	2.80 ± 0.22[b]
Formate (g L^{-1})	0.26 ± 0.02[a]	0.27 ± 0.02[a]	0.23 ± 0.04[b]
Acetate (g L^{-1})	1.37 ± 0.19[a]	1.40 ± 0.09[a]	1.08 ± 0.32[b]
HA average MW (g L^{-1})	3.09 ± 0.17[a]	3.50 ± 0.19[b]	3.60 ± 0.20[b]

Xf – Final cell mass in inocula; HA – Hyaluronic acid; YP/X – HA production per cell growth; YP/S – HA production per glucose consumption; YX/S – Cell growth per glucose consumption; MW – Molecular weight.
Different lower-case letters indicate statistical difference at $\alpha = 0.05$ level in each column. Values with the same letters in the same parameters indicate that the values did not differ by the Tukey test at 0.95 confidence interval.

the microbial production of HA contain glucose as a carbon source (Armstrong, 1997; Chong and Nielsen, 2003), and amounts of complex nitrogen sources like yeast extract, peptones, casein hydrolysate, beyond growth factors such as magnesium and phosphates (Armstrong, 1997). The amounts of the nutrients are varied as reported in the literature. In this work, we used the ratio 7.8:1 (glucose:nitrogen) according to our previous studies with agricultural resources derivatives (Pires et al., 2010a,b; Pires and Santana, 2010). In Petri dishes, our previous results showed that the counts of *S. zooepidemicus* were similar to control (BHI with 5% sheep blood), only when 67 g L^{-1} of SP was used (PII inoculum). This concentration is twice that of BHI (37 g L^{-1}).

4. Conclusions

The animal nutrient sources BHI (37 g L^{-1}) and 5% v/v sheep blood in the first seed in solid culture medium can be totally replaced by 67 g L^{-1} soy peptone, for inoculum preparation of *S. zooepidemicus*. This replacement preserves the HA produced by fermentation of contaminations from animal sources, and also benefits pharmaceutical and cosmetic applications.

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