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Conversion of cheese whey into a fucose- and glucuronic acid-rich extracellular polysaccharide by *Enterobacter* A47

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ABSTRACT

Cheese whey was used as the sole substrate for the production of extracellular polysaccharides (EPS) by *Enterobacter* A47. An EPS concentration of 6.40 g L^{-1} was reached within 3.2 days of cultivation, corresponding to a volumetric productivity of 2.00 g L^{-1} d^{-1}. The produced EPS was mainly composed of glucuronic acid (29 mol%) and fucose (29 mol%), with lower contents of glucose and galactose (21 mol% each) and a total acyl groups content of 32 wt.%. The polymer had an average molecular weight of 1.8×10^6 Da, with a polydispersity index of 1.2, and an intrinsic viscosity of 8.0 dL g^{-1}. EPS aqueous solutions (1.0 wt.% in 0.01 M NaCl, at pH 8.0) presented a shear thinning behavior with a viscosity of the first Newtonian plateau approaching 0.1 Pa s. This novel glucuronic acid-rich polymer possesses interesting rheological properties, which, together with its high content of glucuronic acid and fucose, two bioactive sugar monomers, confers it a great potential for use in high-value applications, such as cosmetics and pharmaceuticals.

1. Introduction

Cheese whey is a lactose-rich byproduct generated in large amounts by the dairy industry, representing about 85–95% of the milk's volume. Worldwide, whey production averages around 1.15×10^8–1.40×10^8 tons per year, with an estimated annual increase of 1–2% (Koller et al., 2012). This byproduct consists mainly of lactose (70–80%), soluble proteins (8–14%), minerals (12–15%), lactic acid (0.8–12%) and fats (1–7%), possessing a high oxygen demand that needs to be treated before discharged into the environment (Koller et al., 2012; Prazeres et al., 2012; Siso, 1996). To overcome this, over the years, several approaches have been proposed aiming to treat or valorize cheese whey. Some of whey products utilization, mostly as food supplements, e.g. sweets, nutraceuticals, additives for processed food and baby food, are limited due to human lactose intolerance (Koller et al., 2012). Additional alternatives for cheese whey's valorization include its use as a biotechnological resource for the generation of added-value products, such as bioethanol (Ozmihci and Kargi, 2007), biogas (Davila-Vazquez et al., 2009), organic acids (Roukas and Kotzekidou, 1998), electricity (Kassongo and Togo, 2010), proteins (Morr and Ha, 1993), polyhydroxyalkanoates (Koller et al., 2012; Pais et al., 2014) and microbial polysaccharides (Fialho et al., 1999; Khanafari and Sepahei, 2007; Savvides et al., 2012).

Bacterial extracellular polysaccharides (EPS) are carbohydrate polymers secreted by the cells that either remain attached to the cell envelope (capsular polysaccharides) or form a slime that is loosely bound to the cell surface (Freitas et al., 2011a; Rehm, 2010). Depending on their subunit composition, structure and molecular mass, EPS can have commercially relevant material properties that are attractive for applications ranging from pharmaceuticals and cosmetics to industrial uses (Prazeres et al., 2012).

Most wild-type EPS producing bacteria are unable to efficiently using lactose as a carbon source because of their low β-galactosidase activity (Siso, 1996). To overcome this issue, it is often necessary to convert the disaccharide lactose into the constituent monosaccharides, glucose and galactose, either chemically or enzymatically, which increases the processes costs. An alternative is the use of genetically modified or adapted bacteria able to utilize lactose as carbon source (Audic et al., 2003). The first hypothesis increase the costs of the process since one additional step is required, while the second has implications on the microorganism

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genetic stability and transfer of recombinant genes with resistance to other bacteria. Thus, it is of interest to find microorganisms that can directly use lactose and convert it into the bioproduct (Davison, 2005).

Enterobacter A47 (DSM 23139) is an EPS-producer that synthesizes high molecular weight heteropolysaccharides composed of fucose, glucose, galactose and glucuronic acid, which present interesting functional properties (Cruz et al., 2011; Dhadge et al., 2014; Ferreira et al., 2014; Freitas et al., 2011b, 2014). *Enterobacter* A47 has demonstrated to be highly versatile due to its ability to use a wide range of substrates, including glycerol, glucose and xylose (Alves et al., 2010; Freitas et al., 2014), but lactose was not previously tested. Hence, in this work, the ability of *Enterobacter* A47 to use lactose and cheese whey as sole carbon sources for EPS production was assessed in fed-batch bioreactor cultivations. The produced polysaccharides were analyzed in terms of sugar and acyl groups composition, average molecular weight and rheological properties.

2. Materials and methods

2.1. Exopolysaccharide production

2.1.1. Microorganism and media

The bacterium *Enterobacter* A47 (DSM 23139) was cryopreserved at −80 °C, in 20% (v/v) glycerol. Reactivation from the stocks culture was performed in Luria Bertani (LB) medium (bactotryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; sodium chloride, 10 g L^{-1}; pH 6.8) that was also the medium used for inocula preparation.

In the bioreactor assays, *Enterobacter* A47 was grown on a slightly modified Medium E* (pH 7.0), with the following composition (per liter): $(NH_4)_2HPO_4$, 3.3 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g; 10 mL of a 100 mM $MgSO_4$ solution and 1 mL of a micronutrient solution (Freitas et al., 2014). The micronutrients solution had the following composition (per liter of 1 N HCl): $FeSO_4{\cdot}7H_2O$, 2.78 g; $MnCl_2{\cdot}4H_2O$, 1.98 g; $CoSO_4{\cdot}7H_2O$, 2.81 g; $CaCl_2{\cdot}2H_2O$, 1.67 g; $CuCl_2{\cdot}2H_2O$, 0.17 g; $ZnSO_4{\cdot}7H_2O$, 0.29 g). Medium E* was supplemented with lactose (Scharlau) or cheese whey (supplied by Lactogal Produtos Alimentares S.A, Portugal) to give a lactose concentration of approximately 70 g L^{-1}. The cheese whey had a lactose content of 78.40 wt.%, as well as 13.62 wt.% protein, 1.21 wt.% fat and 0.89 wt.% lactic acid. Concentrated lactose and cheese whey aqueous solutions (125 g L^{-1}) were prepared and autoclaved separately (121 °C, 2 atm, for 30 min). The autoclaved cheese whey mixture was further centrifuged, under aseptic conditions, at 10,375 × *g* during 15 min, to separate undissolved constituents and precipitated proteins. The resulting lactose-rich supernatant was used to supplement the cultivation medium.

2.1.2. Bioreactor cultivation

The experiments were performed in 5 L bioreactors (BioStat B, Sartorius, Germany) with initial working volumes of 2.5 L. Inocula for the assays were prepared by inoculating 20 mL of LB medium grown cells into 200 mL fresh LB medium and incubation in an orbital shaker for 65 h (at 30 °C, and 200 rpm). The culture obtained was centrifuged (4053 × *g*, for 5 min) and the cells were resuspended in 50 mL phosphate buffer (K_2HPO_4, 5.8 g L^{-1}; KH_2PO_4, 3.7 g L^{-1}; pH 7.0) for inoculation in the bioreactor.

All assays took 4 days, under controlled temperature and pH conditions of 30 ± 0.2 °C and 7.0 ± 0.02, respectively. The aeration rate (0.125 vvm – volume of air per volume of reactor per minute) was kept constant throughout all cultivation runs. The dissolved oxygen level (DO) was controlled below 10% by the automatic variation of the stirrer speed (300–800 rpm). In the fed-batch phase, the bioreactor was fed with Medium E* supplemented with either lactose or cheese whey (lactose concentration of ∼120 g L^{-1}) at a constant lactose feeding rate of 5 g L^{-1} h^{-1}. Samples (25 mL) were periodically withdrawn from the bioreactor for quantification of biomass, lactose, ammonium and EPS concentration, as well as measurement of the broth's viscosity.

2.1.3. Analytical techniques

The viscosity of culture broth samples was measured immediately after collection from the bioreactor, using a digital viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA), for shear rates between 0.28 s^{-1} and 55.8 s^{-1}.

Culture broth samples were centrifuged at 13,000 × *g*, for 15 min, for cell separation. Viscous samples (>100 cPs) were diluted (1:2, v/v) with deionized water for viscosity reduction. The cell-free supernatant was used for the determination of nutrients concentration, and for EPS quantification. The cell pellet was used for the gravimetric determination of the cell dry weight (CDW), after washing twice with deionised water (resuspension in water, centrifugation at 13,000 × *g*, for 15 min, and, finally, resuspension in deionised water) and drying at 100 °C until constant weight.

Sugars (lactose, glucose and galactose) and organic acids (citric and lactic acids) in the cell-free supernatant samples were determined by high performance liquid chromatography (HPLC), with a MetaCarb 87H column (Varian) coupled to a refractive index detector. The analysis was performed at 50 °C, with sulphuric acid (0.01 N) as eluent, at a flow rate of 0.6 mL min^{-1}. Lactose (Scharlau), glucose (Sigma), galactose (Alfa Aesar), citric acid (Panreac) and lactic acid (Sigma) were used as standards at concentrations between 0.062 g L^{-1} and 1.0 g L^{-1}. Ammonium concentration was determined using a potentiometric sensor (Thermo Electron Corporation, Orion 9512). Ammonium chloride (Sigma) was used as standard at concentrations between 0.006 g L^{-1} and 1.8 g L^{-1}.

The EPS was extracted from the cultivation broth by the procedure described by Freitas et al. (2014), with minor modifications. Briefly, the cell-free supernatant was subjected to thermal treatment (70 °C, during 30 min) to denature remaining cheese whey and/or bacterial proteins. The precipitated proteins were removed by centrifugation (13,000 × *g*, for 15 min) and the treated supernatant was dialyzed with a 10,000 MWCO membrane (SnakeSkin™ Pleated Dialysis Tubing, Thermo Scientific), against deionized water, over 48 h at 4 °C, and, finally, freeze dried. A cheese whey solution (125 g L^{-1}) was subjected to the same procedure to quantify the content in high molecular weight compounds present in the cheese whey powder.

All analyses were performed in duplicate.

2.1.4. Calculations

The overall volumetric EPS productivity (r_p, g L^{-1} d^{-1}) was determined as follows:

$$r_p = \frac{\Delta EPS}{\Delta t} \quad (1)$$

where ΔEPS is the concentration of EPS produced (g L^{-1}) within the time period Δt (d).

2.2. Polymer characterization

2.2.1. Chemical composition

For the EPS compositional analysis, polymer samples (∼5 mg) dissolved in deionized water (5 mL) were hydrolyzed with trifluoroacetic acid (TFA) (0.1 mL TFA 99%) at 120 °C, for 2 h. The hydrolysate was used for the identification and quantification of the constituent monosaccharides by HPLC, using a CarboPac PA10 column (Dionex), equipped with an amperometric detector (Dionex), as described by Freitas et al. (2014). The acid hydrolysates were also used for the identification and quantification of acyl groups by