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Kinetics of production and characterization of the fucose-containing exopolysaccharide from *Enterobacter* A47

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ABSTRACT

A fucose-containing exopolysaccharide (EPS) was produced by the bacterium *Enterobacter* A47 using glycerol byproduct from the biodiesel industry. The analysis of kinetic data suggested a partially growth associated EPS synthesis model. Although the EPS was composed of fucose, galactose and glucose at all cultivation stages, their relative proportion has varied considerably during the run. At the beginning (24 h), glucose was the main component (82.4 wt.%), being fucose and galactose minor components (5.0 wt.% and 10.9 wt.%, respectively), while at the end (96 h) it was composed of 26.0 wt.% fucose, 28.9 wt.% galactose and 43.7 wt.% glucose. The acyl groups content and composition have also changed, reaching their maximum content (19.2 wt.%) at the end of the run. Moreover, the molecular weight has increased linearly during the run (from 8×10^5 to 5×10^6). The changes observed in EPS composition and molecular weight have also had an impact upon the polymer's intrinsic viscosity, as shown by its linear increase from 3.95 to 10.72 dL g^{-1}. The results suggest that the culture might have synthesized at least two distinct EPS, with different sugar composition and average molecular weight, which predominated at different cultivation stages.

1. Introduction

Polysaccharides' distinct physical–chemical properties, such as water retention capacity, rheology (e.g. emulsifying, thickening and gelling agents) and film forming capacity, allow their application on several industries (e.g. food, cosmetic and pharmaceutical) (Moreno et al., 1998). Plants, crustacean, algae, as well as a wide range of microorganisms, represent sources of a wide diversity of natural polysaccharides. In the last years, the interest on this sort of polymeric biomaterials has increased due to their environmentally friendly features as biodegradable, biocompatible and value-added products (Kumar et al., 2007).

Microorganisms usually have higher growth rates than plants, crustacean and algae, being microbial production of polysaccharides more amenable to process manipulation, allowing for improved yields, productivity and properties (Alves et al., 2010). On the other hand, industrial microbial production is limited by the high cost of the most commonly used carbon sources (e.g. glucose, fructose, sucrose) (Kumar et al., 2007). This limitation can be overcome by replacing those traditional substrates by low cost carbon sources, such as agro and industrial wastes or byproducts (Kumar et al., 2007). Several industrial processes, essentially biodiesel production, generate large quantities of glycerol as byproduct. Since this glycerol contains several impurities, it cannot be used in many of the traditional glycerol applications unless costly purification steps are performed (Freitas et al., 2009). Hence, it is necessary to develop alternative processes to convert this crude glycerol, into higher value products. The use of glycerol byproduct as carbon source for microbial cultivations may contribute for the reduction of production costs, thus making those bioprocesses more cost effective.

Microbial polysaccharides can be divided into intracellular, structural and extracellular polysaccharides. Extracellular polysaccharides or exopolysaccharides (EPS) are secreted by the cells, either as a capsule that remains associated with the cell surface or as a slime which is loosely bound to the cell surface (Kumar et al., 2007). EPS have easier extraction processes, which is an advantage comparing to other natural polysaccharides (e.g. plants or algae cell-wall constituents).

EPS are composed of monomers such as neutral sugars and/or acidic or amino-sugars, commonly containing non-sugar components, such as acyl groups (e.g. acetyl, pyruvil, succinyl). The most common sugar residues in EPS structures are glucose and galactose. However, certain EPS have an increased value due to their content

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Nomenclature

S	Glycerol concentration ($g\,L^{-1}$)
N	Nitrogen concentration ($g\,L^{-1}$)
X	Biomass concentration ($g\,L^{-1}$)
P	Concentration of exopolysaccharide ($g\,L^{-1}$)
$Y_{X/S}$	Yield of biomass on glycerol ($g\,g^{-1}$)
$Y_{P/S}$	Yield of exopolysaccharide on glycerol ($g\,g^{-1}$)
$Y_{X/N}$	Yield of biomass on nitrogen ($g\,g^{-1}$)
D	Dilution rate (h^{-1})
V	Volume (L)
K_S	Glycerol half saturation constant ($g\,L^{-1}$)
K_N	Nitrogen half saturation constant ($g\,L^{-1}$)
m_S	Maintenance coefficient on glycerol ($g\,g^{-1}\,h^{-1}$)
α	Yield of product synthesis per biomass produced ($g\,g^{-1}$)
β	Specific rate of non-growth associated product synthesis ($g\,g^{-1}\,h^{-1}$)
μ	Specific rate of biomass growth (h^{-1})
μ_{max}	Maximum specific rate of biomass growth (h^{-1})
ν_S	Specific rate of glycerol uptake ($g\,g^{-1}\,h^{-1}$)
ν_N	Specific rate of nitrogen uptake ($g\,g^{-1}\,h^{-1}$)
ν_P	Specific rate of exopolysaccharide production ($g\,g^{-1}\,h^{-1}$)

in some rare sugars, which occur rarely in Nature (Vanhooren and Van Damme, 2000). One of those rare sugars is fucose. It has been reported that fucose-containing polysaccharides possess biological activity that potentiate their use in medical or cosmetic areas, for example, as anti-carcinogenic and anti-inflammatory agents or as moisturizing and anti-aging additives, respectively (Cescutti et al., 2005; Péterszegi et al., 2003; Guetta et al., 2003).

Fucose-containing EPS have been reported to be produced by several bacterial genera, including *Klebsiella*, *Clavibacter*, *Escherichia* and *Enterobacter*. Examples of *Enterobacter* fucose-containing EPS producing strains include: *Enterobacter amnigenus* that produces a heteropolymer containing glucose, galactose, fucose, mannose, glucuronic acid and pyruvil (Cescutti et al., 2005); *Enterobacter* sp. that secretes an acidic EPS in which glucose, mannose, rhamnose and fucose monomers are present in a molar ratio of 3.3:3.0:2.6:1 (Shimada et al., 1997); and *Enterobacter cloacae* that produces an EPS containing glucose, galactose, glucuronic acid, fucose and acetyl in the molar ratio of 5:4:4:11:1 (Meade et al., 1994).

Formerly, we have reported that in a nutrient medium containing glycerol byproduct from the biodiesel industry as the sole carbon source, the bacterium *Enterobacter* A47 (DSM 23139) produced an EPS composed of fucose, glucose and galactose (Alves et al., 2010). A preliminary polymer characterization in terms of its chemical composition, molecular weight and intrinsic viscosity was performed (Freitas et al., 2011). In this work, we characterize cell growth and EPS synthesis kinetics with the aid of a simple kinetic model. Furthermore, the progress of the polymer's chemical composition, molecular weight and intrinsic viscosity along the cultivation run was analyzed with the objective of describing the behavior of the culture during its growth on glycerol and EPS synthesis.

2. Materials and methods

2.1. Fucose-containing EPS production

2.1.1. Microorganism and media

Enterobacter A47 (DSM 23139) (Freitas et al., 2011) was grown on a slightly modified Medium E* (pH 7.0), supplemented with glycerol byproduct to give a concentration between 25 and 50 $g\,L^{-1}$, as described by Freitas et al. (2009). Glycerol byproduct (with a glycerol content ca. 89%) was supplied by SGC Energia, SGPS, SA, Portugal.

Inoculums for bioreactor experiments were prepared by incubating the culture in Medium E* supplemented with glycerol byproduct (40 $g\,L^{-1}$), in shake flasks, for 72 h at 30 °C, in an incubator shaker (150 rpm).

2.1.2. Bioreactor operation

The 2 L bioreactor (BioStat B-plus, Sartorius) containing 1.3 L of Medium E* (Freitas et al., 2009) supplemented with glycerol byproduct (concentration ca. 40 $g\,L^{-1}$) was inoculated with the culture (400 mL). The bioreactor was operated as described by Freitas et al. (2011). Briefly, it was operated in a batch mode during the first day of cultivation and, in a fed-batch mode, for the next three days, by supplying the bioreactor with cultivation Medium E*, with a glycerol concentration of 200 $g\,L^{-1}$, at a constant rate of 4.5 $mL\,h^{-1}$. Temperature and pH were controlled at 30 ± 0.1 °C and 7.00 ± 0.05, respectively. The aeration rate (0.125 vvm, volume of air per volume of reactor per minute) was kept constant throughout the cultivation, and the dissolved oxygen concentration (DO) was controlled by automatic variation of the stirrer speed (200–800 rpm) provided by two 6-blade impellers. During the fed-batch phase, the DO was maintained below 10%.

2.1.3. Analytical techniques

Culture broth samples were centrifuged at 13,000 × *g*, for 15 min, for cell separation. The cell-free supernatant was stored at −20 °C for the determination of glycerol and ammonium concentrations, and for the quantification of the EPS produced. The cell pellet was used for the gravimetric determination of the cell dry weight (CDW), after washing with deionized water (resuspension in water, centrifugation at 13,000 × *g*, for 10 min, and, finally, resuspension in water and filtration through 0.20 μm filters).

Glycerol concentration in the cell-free supernatant was determined by high performance liquid chromatography (HPLC) with an Aminex HPX-87H column (BioRad), coupled to a refractometer. The analysis was performed at 50 °C, with sulphuric acid (H_2SO_4 0.01 N) as eluent, at a flow rate of 0.6 $mL\,min^{-1}$. Ammonium concentration was determined using a potentiometric sensor (Thermo Electron Corporation, Orion 9512). The viscosity of the culture broth samples was measured using a controlled stress rheometer (Haake RS-75, Germany) equipped with a cone and plate geometry (diameter 3.5 mm, angle 2°), as described by Alves et al. (2010).

2.1.4. EPS extraction

The culture broth recovered from the bioreactor at the end of the cultivation was diluted with deionized water (1:2, v/v) for viscosity reduction and the bacterial cells were removed by centrifugation (13,000 × *g*, 1 h). The cell-free supernatant was subjected to thermal treatment (70 °C, 1 h) to inactivate bacterial enzymes that might cause polymer degradation during the subsequent purification steps. The treated supernatant was centrifuged (13,000 × *g*, 1 h) to remove any remaining cell debris and denatured proteins. Finally, it was dialyzed with a 10,000 MWCO membrane (SnakeSkin™ Pleated Dialysis Tubing, Thermo Scientific), against deionized water (48 h, 4 °C) and freeze dried.

2.2. Analysis and characterization of process kinetics

A simple mathematical model was employed for cell growth and product synthesis kinetics characterization. Growth kinetics