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Controlled production of polysaccharides–exploiting nutrient supply for levan and heteropolysaccharide formation in *Paenibacillus* sp.

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ABSTRACT

Bacterial exopolysaccharides (EPSs) are promising sustainable alternatives to synthetic polymers. Here we describe the production and characterization of different EPSs produced by the recently isolated *Paenibacillus* sp. 2H2. A final EPS titer of 4.54 g L^{-1} was recovered after a 17-h fermentation, corresponding to a volumetric productivity of 0.27 g L^{-1} h^{-1}. Remarkably, supplying the fermentation with specific carbon and nitrogen sources could be exploited for the production of different polymers. A pure heteropolysaccharide composed of glucose, mannose, galactose, and glucuronic acid (3.5:2:1:0.1) was obtained when using glucose/glycerol and peptone as substrates. A pure levan-type polymer or mixture of both polymers was observed with sucrose and $NaNO_3$ or peptone. To our knowledge, this is the first report that nutrients, particularly nitrogen sources, can be used to fine-tune EPS production in *Paenibacillaceae*. Rheological characterization of the heteropolysaccharide revealed impressive thickening properties, suggesting its potential application in commodity materials.

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1. Introduction

Polysaccharides are a very versatile class of natural, non-toxic, and biodegradable polymers, produced by every domain of life, and occur in a huge number of variations. In the natural environment, their versatility is reflected by the numerous tasks they fulfill. Polysaccharides serve, as structural elements, nutrient reservoirs, protection against external stressors, and virulence factors (Sutherland, 1998). This physicochemical variability is imparted by variations in molecular weight, sugar monomer composition, and side chains. Industry discovered the unique properties of polysaccharides years ago. Several products have already been brought to market for food, feed, or cosmetic applications; enhanced oil recovery; and drug delivery (Freitas, Alves, & Reis, 2014; Garcıía-Ochoa, Santos, Casas, & Gómez, 2000; Raveendran, Poulose, Yoshida, Maekawa, & Kumar, 2013; Suresh Kumar, Mody, & Jha, 2007). Polysaccharides are also used as rheological additives, stabilizers, emulsifiers and flocculants (Schmid, Sperl, & Sieber, 2014). Prominent examples of useful polysaccharides include plant- or algae-derived galactomannans and carrageenans; fungal schizophyllan, scleroglucan, and pullulan; and bacterial xanthan and levan, as well as the class of sphingans. However, synthetic, petrochemical-based polymers still outperform natural polymers in terms of functionality and profitability in many applications (Rehm, 2010). Global megatrends, such as resource efficiency and corporate social responsibility, put pressure on the market and drive the search for novel natural polysaccharides to replace undesired petrochemical substances, like polyacrylates, polyvinyl polymers, and polyacrylamides, especially in consumer-oriented commodities.

Bacterial polysaccharides represent the most promising resource for natural alternatives to synthetic polymers. Over 400 bacterial polysaccharides have been structurally characterized (Rühmann et al., 2015b; Toukach, Joshi, Ranzinger, Knirel, & von der Lieth, 2007); more reports on other prokaryotic polysaccharides can be found in the literature. Considering that up to <1% of bacteria are culturable (Stewart, 2012), many bacterial polysaccharides have not yet been identified. Furthermore, bacterial polysaccharides can be produced independently of season and location. Exopolysaccharides (EPSs) are secreted into the extracellular space, facilitating downstream processing and reducing production costs. Recent advances in genome editing tools have significantly simplified bacterial genome targeted alteration, bringing tailored EPSs within researchers' grasps (Jiang, Bikard, Cox, Zhang, & Marraffini, 2013; Peters et al., 2015). Novel screening methodologies have

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been developed that enable high-throughput analyses of hundreds of strains per day and facilitate new, interesting EPS identification (Rühmann et al., 2015a).

The *Paenibacillus* genus provides one example of the intense research on new microbial EPSs. Several recent studies have identified novel EPSs produced by *Paenibacillus* isolates. A review article summarized the state of the art with respect to *Paenibacillus* EPSs (Liang & Wang, 2015). The habitats of this Gram-positive genus include soils, marine sediments, the rhizospheres of various crop plants, insect larvae, and forest trees (Raza, Yang, & Shen, 2008). Although 89 different *Paenibacillus* species are known so far, reports on EPS formation are limited to a very small number of strains, mainly those belonging to the plant growth-promoting species *Paenibacillus polymyxa* or phylogenetically closely related representatives, wherein EPSs were shown to be important for biofilm formation and plant root colonization (Haggag, 2007; Timmusk, Grantcharova, & Wagner, 2005). Structural differences among characterized *Paenibacillus* EPSs, however, are anything but small. Descriptions range from fructose-containing levan-type polysaccharides (Han & Clarke, 1990; Liu et al., 2009) to beta-glucans (Jung et al., 2007; Rafigh, Yazdi, Vossoughi, Safekordi, & Ardjmand, 2014) to complex heteropolysaccharides comprising different sugars and substituents, including glucose, galactose, mannose, rhamnose, xylose, sorbose, fucose, fructose, pyruvate, and uronic acids (Lee et al., 1997; Li et al., 2013; Madden, Dea, & Steer, 1986; Morillo, Guerra del Águila, Aguilera, Ramos-Cormenzana, & Monteoliva-Sánchez, 2007; Raza, Makeen, Wang, Xu, & Qirong, 2011; Tang et al., 2014; Yegorenkova, Tregubova, Matora, Burygin, & Ignatov, 2008). Some authors describe the existence of two/three polymer fractions of different molecular weights, charges, and monomer compositions. (Liu et al., 2009; Madden et al., 1986; Morillo et al., 2007; Tang et al., 2014).

Reasons for EPS versatility among strains may be found at the genomic level, in the EPS biosynthesis machinery, the demanding analytics of EPS characterization which potentially yield differing results, or the applied process parameters, such as the carbon and nitrogen sources used during fermentation. Polysaccharide formation is favored by high carbon to nitrogen ratios. Furthermore, levansucrases, which are frequently associated with *P. polymyxa,* catalyze a transfructosylation reaction when sucrose is present, resulting in microbial levan formation (Han & Clarke, 1990; Lee et al., 1997). Besides this, very little information is available on the impact of nutrient composition on EPS formation in *Paenibacillaceae*. Particularly, the influence of different nitrogen sources has not been described in detail, and variations in EPS structure, molecular weight, and product titer have been understood as strain specificities rather than the result of process parameters.

Here we describe the fermentative production of EPSs by a novel *Paenibacillus* sp. strain. The influence of different carbon and nitrogen sources on polymer biosynthesis and EPS composition is investigated, and two different analytical methods for the monomer analysis of purified EPSs are validated. Furthermore, rheological properties of isolated EPSs are described. The results suggest that the nitrogen source plays a key role in EPS regulation in *Paenibacillaceae* and emphasize the importance of sophisticated, in-depth analysis of purified EPSs for reliable product characterization.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were purchased in analytical or microbiological grade from Sigma-Aldrich, Carl Roth GmbH (Karlsruhe, Germany) or Merck KGaA (Darmstadt, Germany) unless otherwise stated. Ultra-pure water was produced using a PURELAB classic system (ELGA LabWater) and used in all experiments.

2.2. Bacterial strain isolation and identification

Paenibacillus sp. 2H2 was identified as a promising EPS producer using a previously described screening technique, selecting for growth characteristics and carbohydrate fingerprint of the polysaccharide produced (Rühmann et al., 2015a). *Paenibacillus* sp. 2H2 was chosen due to its interesting carbohydrate profile and high broth viscosity.

To identify the phylogenetic affiliation of the strain, genomic DNA was isolated from a pure culture and 16S rDNA was amplified and sequenced using primers 27fn (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525r (5′-AAGGAGGTGWTCCARCC-3′). Sequence data was aligned using Clone Manager 9 Professional software, and phylogenetic neighbors were identified using the BLASTN program against the curated EzTaxon-e database (Kim et al., 2012). Phylogenetic tree was constructed using MEGA version 6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) applying the neighbor joining method.

2.3. Culture conditions and fed-batch fermentation

Flask cultures were prepared in baffled 500-mL shake flasks sealed with aluminum caps. The flasks contained 100 mL basal MM1 P100 medium carbon and nitrogen sources, 1.33 g L^{-1} magnesium sulfate heptahydrate, 1.67 g L^{-1} potassium dihydrogen phosphate, 0.05 g L^{-1} calcium chloride dihydrate, 2 mL L^{-1} RPMI 1640 vitamins solution (Sigma-Aldrich) and 1 mL L^{-1} trace elements solution (2.5 g L^{-1} iron(II) sulfate heptahydrate, 2.1 g L^{-1} sodium tartrate dihydrate, 1.8 g L^{-1} manganese(II) chloride tetrahydrate, 0.075 g L^{-1} cobalt(II) chloride hexahydrate, 0.031 g L^{-1} copper(II) sulfate heptahydrate, 0.258 g L^{-1} boric acid, 0.023 g L^{-1} sodium molybdate and 0.021 g L^{-1} zinc chloride). The initial pH of the medium was adjusted to 7. Different carbon and nitrogen sources were tested (30 g L^{-1} sucrose, glucose or glycerol; 0.05 mol L^{-1} nitrogen from either peptone or $NaNO_3$). Cultures were inoculated with a single colony and incubated at 30 °C and 150 rpm for 48 h.

In-depth characterization of microbial growth and fermentative EPS production was conducted at 30 °C in a 30-L BIOSTAT® Cplus bioreactor (Sartorius AG, Göttingen, Germany), equipped with three propeller stirrers attached to one agitator shaft. A 20-L MM1 P100 medium containing 36 g L^{-1} glucose as carbon and 5 g L^{-1} peptone as nitrogen sources was used. The main culture was inoculated with an overnight seed culture (1 L MM1 P100 in a 5-L baffled shake-flask) at 5% of the final fermentation volume. Culture pH was kept constant at 6.8 with 42% H_3PO_4 and 8.4 M NaOH. The dissolved oxygen content was regulated via a cascade of stirrer speed and gas flow. Oxygen saturation was determined at maximal stirring (400 rpm) and gas flow (1.5 vvm) before inoculation. During the fermentation, 30% of the previously defined O_2 saturation value was initially maintained by increasing stirrer speed and by enhancing gas flow. CO_2 and O_2 concentrations in the exhaust gas were monitored using BCP-CO2 and BCP-02 sensors, respectively (BlueSens gas sensor GmbH, Herten, Germany). A further 10 g L^{-1} glucose was fed between hour 10 and 13 by adding a 360-g L^{-1} glucose solution at a rate of 3 mL min^{-1}. The process was stopped after 20 h. Samples were obtained every 3 h to determine broth viscosity, optical density (OD) at 600 nm, glucose concentration, protein content, EPS titer, and EPS molecular weight. For titer measurements, 30-g culture broth was treated according to EPS purification scheme of the shake-flask experiments described below.

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