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Development and validation of a colorimetric assay for simultaneous quantification of neutral and uronic sugars

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ABSTRACT

A colorimetric assay based on the conventional anthrone reaction was investigated for specific quantification of uronic acids (UA) in the presence of neutral sugars and/or proteins. Scanning of glucose (Glu) and glucuronic acid (GlA) was performed after the reaction with anthrone and a double absorbance reading was made, at 560 nm and at 620 nm, in order to quantify the UA and neutral sugars separately.

The assay was implemented on binary or ternary solutions containing Glu, GlA and bovine serum albumin (BSA) in order to validate its specificity towards sugars and check possible interference with other biochemical components such as proteins. Statistical analysis indicated that this assay provided correct quantification of uronic sugars from 50 to 400 mg/l and of neutral sugars from 20 to 80 mg/l, in the presence of proteins with concentrations reaching 600 mg/l.

The proposed protocol can be of great interest for simultaneous determination of uronic and neutral sugars in complex biological samples. In particular, it can be used to correctly quantify the Extracellular Polymeric Substances (EPS) isolated from the biological matrix of many bacterial aggregates, even in the presence of EPS extractant such as EDTA.

1. Introduction

Microbial aggregates like flocs, granules or biofilms are found in various natural environments such as soil, water, sediments or activated sludge. In wastewater systems, activated sludges are beneficial since they take part in self-purification processes. However, some microbial aggregates such as biofilms can have detrimental effects due to biocorrosion or biofouling of industrial units (Denkhaus et al., 2007).

Microbial aggregates are composed of bacterial cells and extracellular material including various extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). In wastewater systems, EPS originate principally from microbial secretion and cell lysis but are also present in the incoming wastewater. The EPS matrix is mainly composed of proteins and polysaccharides while lipids and nucleic acids are rather found in minor proportions (Azero et al., 1999; Ras et al., 2011). EPS have been shown to play diverse important roles in the structure and functions of microbial aggregates, such as involvement in adhesion and bioaggregation processes or in nutrient transfer limitations (Ahimou et al., 2007; Caudan et al., 2012; Denkhaus et al., 2007). In consequence, during the two last decades, many studies have dealt with EPS quantification and characterization, but the EPS matrix still remains a "dark matter" which needs further elucidation.

In general, bioflocs and biofilms have a global negative charge due to chemical functions present on the bacterial cells and EPS (Sobeck and Higgins, 2002). Proteins are composed of amino acids, some of them, e.g. glutamic or aspartic acids, containing free carboxyl groups which have a negative charge

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under neutral and alkaline operating conditions. Polysaccharides composition depends on bacteria species and can contain neutral monomers and/or uronic acids (UA), such as alginate. UA correspond to a partially oxidized form of osidic monomers bearing at least one carboxylic function. These anionic polymers can promote bioflocculation through ionic interactions with divalent cations, thus becoming involved in the aggregation and stability of the EPS matrix (Sobeck and Higgins, 2002). In multi-species aerobic granules used for both carbon and nitrogen elimination, Caudan et al. (2012) have shown that divalent cation bridging specifically concerns anionic proteins. In addition, Lin et al. (2010) have shown that Ca^{2+} has a strong capacity to bind with an alternating mannuronate–guluronate alginate-like exopolysaccharide extracted from aerobic granular sludges. Disrupting these bridges is thus an interesting way to destroy the aggregates and extract the EPS, which is why various chemicals targeting ionic linkages have been tested (Sheng et al., 2010). EDTA has been found to be particularly efficient for cation chelation and for extraction of large amounts of EPS, without generating cell lysis (D'Abzac et al., 2010; Liu and Fang, 2002; Ras et al., 2008a). However, residual EDTA often contaminates the EPS extracts obtained, and may then interfere with EPS colorimetric quantification (D'Abzac et al., 2010; Bourven et al., 2012).

Regarding the colorimetric determination of total carbohydrates (reducing and non-reducing sugars), Scheng et al. (2010) reported that the methods generally used are the phenol-sulfuric acid method (Dubois et al., 1956) or the anthrone method (Dreywood, 1946). These assays are based on a first polymer acid hydrolysis, followed by intramolecular dehydration of all the osidic monomers in acidic conditions. The furfural derivatives thus obtained condense on phenol or anthrone to form chromogenic products which absorb at 490 and 620 nm respectively. In the literature, EPS of the bacterial systems studied essentially contain hexoses and glucose is usually used as a standard (Verhoef et al., 2005).

Considering the quantification of total neutral sugars with phenol, the 485 nm wavelength is usually used but the absorbance values measured vary strongly with the monosaccharide composition (Dubois and al., 1956). In consequence, the phenol method can underestimate or overestimate the total neutral sugars.

For the quantification of neutral sugars with anthrone, the yellow absorbance is measured at 620 nm. As with Dubois' method, not all carbohydrates form colour complexes with the same intensity. The intensities of heptose and pentose absorbances are lower than those of hexose. In this case, the heptoses and pentoses are underestimated. For example, xylose gives absorbance levels of 10% compared to glucose equivalent (Laine et al., 1981). Since the bacterial systems studied essentially contained hexoses, the anthrone assay could, nevertheless, be considered as a correct way to quantify total neutral sugars.

Colorimetric methods for assaying sugar in complex biological samples remain problematic because of the presence of numerous biochemical species that can interfere with sugar quantification. These interferences depend strongly on the protocols and reactants used. The anthrone method is more suitable than the phenol one for quantifying total carbohydrates in extracts of bacterial origin contaminated by nucleic acids. However, except when considerable cell lysis has occurred, these components are generally minor in the bacterial aggregate extracts (Avella et al., 2010). Attention should rather be focused on proteins and UA, which are among the main components of EPS, depending on the origin of the aggregates and the extraction protocols used (Ras et al., 2008a; Lin et al., 2010). Whether the phenol-sulfuric acid or the anthrone methods are considered, proteins do not significantly interfere with them (Gaudy, 1962).

The EPS matrix of some biofilms is mainly composed of UA (Rättö et al., 2006) and recent studies have indicated that UA can also be extracted from multi-species granular sludges (Lin et al., 2010; Seviour et al., 2012). Because of their similar chemical structure to that of neutral sugars, UA may give a large absorbance signal at 485 nm or 620 nm when assayed with the phenol or the anthrone assay respectively. In the case of phenol assay, uronic sugars absorb at 485 nm with absorbance values ranging from 42% to 63% compared to the absorbance of glucose at the same concentration. Their reaction with the anthrone reactant is lower, however, leading to absorbance values less than 20% of glucose equivalent (Kunerth and Youngs, 1984). Consequently, for UA-containing samples, these colorimetric methods involve either an underestimation or an overestimation of the total neutral sugar concentrations.

The UA content of biological samples was originally estimated by decarboxylation procedures based on the quantitative conversion of UA into CO_2. Due to interference with amino sugars and proteins, it has been replaced by colorimetric methods such as Dische's method (Dische, 1947) and Blumenkranz and Asboe–Hansen's method (Blumenkranz and Asboe-Hansen, 1973), using carbazole or metahydroxydiphenyl, (MHDP), respectively. Dische's method has been criticized for its lack of specificity, and in particular for its interference with neutral sugars (Filisetti-Cozzi and Carpita, 1991). As an alternative to carbazole, MHDP has been claimed to be more sensitive and specific and also to be simpler and quicker to use. However, concentrations of non-uronide sugar above 200 mg/l lead to an overestimation of the UA concentrations (Kintner and Van Buren, 1982). Finally, the existing assays for UA quantification are not suited to complex environments, either because of interference with proteins reported in the decarboxylation assays or because of interferences with neutral sugars reported in the carbazole and MHDP assays.

In this study, a new procedure is proposed for the specific quantification of UA in the presence of neutral sugars and/or proteins. The procedure is based on the reaction of anthrone with both neutral and UA sugars but a double absorbance reading is proposed, at 560 nm and at 620 nm, in order to quantify the UA and neutral sugars separately. The sensitivity and the detection parameters were checked with pure solutions of glucose and glucuronic acid (GlA). Then, the assay was implemented with binary or ternary solutions containing glucose, GlA and bovine serum albumin (BSA) in order to validate its specificity towards sugars and check possible interferences with other biochemical components such as proteins. An experimental protocol is proposed for simultaneous quantification of neutral and uronic sugars in complex biological samples.