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Colorimetric measurement of carbohydrates in biological wastewater treatment systems: A critical evaluation

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ABSTRACT

Four laboratory preparations and three commercially available assay kits were tested on the same carbohydrate samples with the addition of 14 different interfering solutes typically found in wastewater treatment plants. This work shows that a wide variety of solutes can interfere with these assays. In addition, a comparative study on the use of these assays with different carbohydrate samples was also carried out, and the metachromatic response was clearly influenced by variation in sample composition. The carbohydrate content in the supernatant of a submerged anaerobic membrane bioreactor (SAMBR) was also measured using these assays, and the amount in the different supernatant samples, with and without a standard addition of glucose to the samples, showed substantial differences. We concluded that the carbohydrates present in wastewater measured using these colorimetric methods could be seriously under- or over-estimated. A new analytical method needs to be developed in order to better understand the biological transformations occurring in anaerobic digestion that leads to the production of soluble microbial products (SMPs) and extracellular polymeric substance (EPS).

1. Introduction

The characterisation of organic matter in wastewater samples has often been limited to lumped parameters such as total organic carbon (TOC), chemical oxygen demand (COD) and biological oxygen demand (BOD), which are carried out using Standard Methods from American Public Health Association (APHA) (Rice, 2012). However, with advances in wastewater treatment in recent decades a more detailed characterisation is required in order to optimise process performance. Since water samples from biological processes contain a wide range of complex organic compounds such as soluble microbial products (SMPs) and extracellular polymeric substances (EPS) (Laspidou and Rittmann, 2002; Ni et al., 2011), the need to chemically identify major specific organic constituents in them is of considerable interest. Several studies have reported that the major organic constituents in SMPs and EPS are proteins-like compounds, polysaccharides (PS)/carbohydrates, humic substances, lipids, and other small molecules (Flemming and Wingender, 2001; Aquino, 2004; Jarusutthirak and Amy, 2006; Zhang et al., 2008; Kunacheva and Stuckey, 2014).

PS are of great importance, and have been extensively studied due to their effect in aerobic granules, biofouling in membrane-related processes, and bio-corrosion (Liu, 2007; Annuk and Moran, 2010; Stuckey, 2010). It has been well-documented that the ability to synthesize and secrete PS is widespread among microorganisms under virtually all physiological conditions (Sutherland, 2007), and various secreted extracellular compounds are often found to be glycosylated even though such compounds are only produced in eukaryotic cells (Upreti et al., 2003). It is also known that these extracellular PS are heavily involved in the formation of three-dimensional structures, and improve the long term stability of aerobic granules (Liu, 2007). Cho and Fane (2002) found that increasing membrane resistance was linked to the increasing production of EPS, and Okamura et al. (2009) suggested that this resistance directly correlates with PS, which undergo intermolecular or intramolecular ionic cross-linking thus clogging the membrane pores and leading to further fouling of the membrane.

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Furthermore, PS-related biofilms of both aerobic and anaerobic microorganisms have been recognised as the most significant contributor to microbially influenced corrosion, which is a major issue for material used in industrial wastewater treatment plant (Beech and Gaylarde, 1999; Pitonzo et al., 2004; Coester and Cloete, 2005).

Although the significant involvement of carbohydrates in these areas of interest has led to the development of various analytical techniques, the most rapid, simple and cheap approach for micro-determination of total carbohydrate concentrations is probably the colorimetric methods. Most colorimetric methods involve reaction of the sample with sulfuric acid and addition of a reagent to develop the chromophore, and were initially developed many decades ago. Several colour developing reagents have been employed, but the most commonly used in wastewater treatment has been anthrone (Dreywood, 1946) and phenol (Du Bois et al., 1951, 1956). Another colorimetric method is the periodic acid-Schiff (PAS) stain, which involves staining macromolecules such as the glycoproteins that contain a large amount of saccharide. This staining method is a common chemical test incorporating Schiff-type reagents that was developed by Hugo Schiff in 1866.

These assays are simple, requiring only chemicals, tubes and a spectrophotometer, and are universally accepted. The reagents can be economically prepared in a laboratory, and a number of assays are available from commercial suppliers such as Sigma-Aldrich, Thermo Scientific, and Abnova. Recently, scaled-down methods have been developed and 96-well microplates have been adapted for higher throughput, and to further economize on reagents (Laurentin and Edwards, 2003; Masuko et al., 2005).

Despite their simplicity, there are two considerations which need to be noted; firstly, it must be emphasized that no single colorimetric method is absolutely specific (Mecozzi, 2005), and hence the results of these assays are often presented in terms of glucose-equivalent concentrations. Such representation has a major drawback where the composition of the carbohydrates are not well known, and variable absorbance responses to different saccharides that are not glucose would very likely be observed. Secondly, there is the problem with interferences (Ashwell, 1957), many of the organics present in wastewater may react with reagents in the presence of strong acids, especially concentrated sulfuric acid, or even simply react with H_2SO_4 alone producing a marked absorption in the ultraviolet region (Dische, 1955). Hence, the carbohydrates measured by these chromogenic methods could be incorrectly estimated.

In addition, a detailed evaluation of these colorimetric saccharide quantification methods is often time consuming, and thus has received limited attention (Raunkjaer et al., 1994; Frøhlund et al., 1996; Jimenez et al., 2013). When using these methods with wastewater it is important to recognize whether or not the methods have a high sensitivity to the different saccharides measured, and/or a low sensitivity to interfering compounds, because wastewater is a relatively complex mixture of different components at different concentrations. Thus there is an urgent need to evaluate these existing assay techniques in some depth, with and without interfering solutes, to see whether they can accurately measure carbohydrates in wastewater. By comparing the performance of these carbohydrate assays simultaneously and comprehensively with a large number of (poly)saccharides, this paper will highlight the need to understand the chemistry and limitations of the colorimetric assays particularly when studying the complex environments in which wastewater is present. It is important to understand that the results are values relative to the amount of glucose, and are not an absolute value. However, in the future it is best to develop new analyses which are simple enough to be used widely, and yet more accurate and informative than the existing assays.

2. Materials and methods

2.1. Reagents and chemicals

All analytical grade chemicals were purchased from Sigma-Aldrich. Sulfuric acid was of ISO grade and was purchased from Merck, while ultrapure water was obtained from a MilliQ water process (Millipore Advantage A10). A 2 mg/mL stock solution of glucose was prepared and stored at 4 °C before use.

2.2. Anthrone method

The reaction of anthrone with saccharides to form a blue-green coloured complex was first reported by Dreywood (1946). Heat and a strongly acidic environment induce both hydrolysis of the glycosidic bonds of polysaccharides, and dehydration of monosaccharides to produce furfural derivatives. These furfuraldehyde compounds react with anthrone producing a coloured product, which is then measured spectrophotometrically. Since Dreywood a number of modifications have been reported to optimise the experimental conditions and improve agreement between the experiential evidence obtained from different carbohydrates (Morris, 1948; Loewus, 1952; Scott and Melvin, 1953; Brooks et al., 1986; Raunkjaer et al., 1994; Frohlund et al., 1996; Laurentin and Edwards, 2003; Rondel et al., 2013).

Since it is photosensitive, and its absorption decreases over time, the anthrone-sulfuric acid reagent is prepared freshly on the day of analysis. One hundred micro liters of a glucose standard, or the sample, is added to separate wells of the same 96-well microplate (Corning), and then 200 μL of 0.1% or 0.2% anthrone in concentrated H_2SO_4 was added to each well. The solution was well mixed using a horizontal shaker for 1 min at room temperature, and the reaction incubated for 30 min at 80 °C in an oven; the microplate was covered and protected from light during the incubation. The plate was then cooled to room temperature before the absorbance was read at 625 nm using a microplate reader (TECAN, Infinite 200 PRO).

2.3. Phenol-sulfuric acid method

Possibly the most reliable and well-known colorimetric assay for carbohydrates is that devised by Du Bois et al. (1951, 1956) utilizing phenol and H_2SO_4. The assay involves mixing carbohydrates and phenol in water, then adding H_2SO_4, and allowing the heat of reaction to drive the dehydration and formation of furfural derivatives that condense with phenols to form orange-yellow complexes. The original method was modified several times, and eventually adapted for 96-well microplates for greater simplicity and speed (Rao and Pattabiraman, 1989; Taylor, 1955; Masuko et al., 2005).

To 50 μL of a glucose standard (0, 4, 8,12,16 and 20 μg/well), or the sample, in the well of a 96-well Corning microplate was added 30 μL of freshly prepared 5% or 8% phenol solution (purified by redistillation). The mixture was shaken for 10 min before 150 μL of concentrated H_2SO_4 was added rapidly; the mixture was then shaken for another 5 min and allowed to stand at room temperature for 25 min. The plate was covered and protected from light during incubation; after incubation the absorbance was read at 490 nm by an Infinite 200 PRO microplate reader (TECAN) with TECAN i-control software.

Two commercially available phenol-sulfuric acid assay kits were purchased from Sigma-Aldrich (MAK104) and Abnova (KA3756). A glucose standard, or a sample, was first added to a series of wells in