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Preliminary Methods for Identification of bacterial alginate or uronic acid containing polysaccharides in Azotobacter and other species

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ABSTRACT:

The aim of this paper is to present the simple techniques employed in detection of uronic acid with rapidity and ease. Many advanced analytical techniques are being employed nowadays with much accuracy but in preliminary studies simple methods are more preferable as it is helpful to give proper direction for future research. Methods discussed in this paper are Carbazole, Blumenkrantz and Asboe-Hansen, UV-vis spectral analysis and TLC (Thin layer Chromatography). Four bacterial cultures (Azotobacter vinelandii, Azotobacter chroococcum, Pseudomonas species and unidentified culture) were studied and the presence of uronic acid was detected in their secreted polysaccharides. Presence of uronic acid was detected by all the methods for all the cultures used except the unidentified culture which showed no results in TLC. From the M:G ratio it is concluded that Azotobacter vinelandii has more guluronic acid whereas Pseudomonas species was found to be showing maximum content of mannuronic acid.

Keywords: Uronic acids, Bacterial alginate, Azotobacter, Pseudomonas,Carbazole, Blumenkrantz & Asboe-Hansen, TLC.

INTRODUCTION:

Uronic acids are the products generated by oxidation of the hydroxyl group of C6 of aldoses. It includes D-mannuronic acid, L-guluronic acid, D-galacturonic acid, L-iduronic acid etc. These are the components of polysaccharides produced by animals, terrestrial plants, seaweeds and

some bacteria [1]. There are a number of polysaccharides which contains uronic acid. Apart from xylan, xanthan, heparin, one such polysaccharide is alginate, it has gained immense attention due to its applications in various food and pharmaceutical industries. They are found to be possessing anti-inflammatory, anticoagulant, antioxidative, immunogenic properties which are essential from medicinal perspective [2]. Alginate is a polysaccharide containing linear copolymers of β-D-mannuronic acid and its C-5 epimer, α-guluronic acid. It is usually obtained from brown seaweeds but can also be produced by Azotobacter and Pseudomonas species [3]. The residues are arranged randomly as MM/MG/GG [4]. Bacterial alginate (Poly M) is usually acetylated at position C-2 and/or C-3 of mannuronate [5,6]. The M/G ratio, molecular weight and other properties are of utmost importance in determining the application of alginate. Alginate oligosaccharides stimulate the growth of human endothelial and keratinocytes cells [7,8]. It was reported by Tomoda et al., 1994 & Hien et al., 2000 [9,10] that oligosaccharides obtained by hydrolysis of alginate can also promote the growth and root elongation in barley and rice. Detection or estimation of uronic acid is of great importance at the preliminary stage of research work if the researcher is interested in studying such polysaccharides. The methods which can be performed with ease along with reliable results are : Blumenkrantz & Asboe-Hansen method, Carbazole method, Spectrophotometric analysis, performing TLC by carrying out partial hydrolysis of polysaccharide. Uronic acid content in the form of galacturonic acid, guluronic acid, mannuronic acid may very well be estimated spectrophotometrically. The best method is by using chromophores to m-hydroxydiphenyl [11,12,13,14], carbazole [15,16] and 3,5-dimethylphenol [17,18]. Lately researchers also performed high performance liquid chromatography resolution [19,20] and the zonal capillary electrophoresis with UV detection. Using m-hydroxydiphenyl as a chromophore [11] has been described as the best quantitative and sensitive method also used in quality control for oligogalacturonide mixtures. The method is based on the formation of pink coloured complex when uronic acid is heated to 100^{o}C in a mixture of sulfuric acid / sodium tetraborate and subsequently treated with m-hydroxydiphenyl. Cesaretti et al., 2003 [21] reported a sensitive and reproducible 96-well assay uronic acid carbazole reaction permitting a rapid processing of samples with less usage of reagents for the determination of complex uronic acid bearing polyanions

like heparin, hyaluronic acid etc. The sensitivity of the reaction was reported to be 1 mg for glucuronic acid and 2 mg for complex polysaccharides as compared to other conventional methods. The spectrophotometric analysis of depolymerized or partially hydrolysed alginates gave maximum absorption around 235 nm [22]. TLC of oligomers can be performed by using butanol /acetic acid / water (4:6:1 v/v) as reported by [23]. Many advanced analytical techniques are employed for further confirmation and analysis like FTIR, GC, GC-MS, HPLC etc. Amongst various uronic acids, D-galacturonic and D-glucuronic acid are in great demand in the pharmaceutical, cosmetic and food industries, its other applications are antioxidants, detoxifying and inactivating agents of various substances in the human body [24], precursors of ascorbic acid [25], and chelating reagents with anti-cancer properties [26]. D-glucuronic acid is also used as a building block of hyaluronic acid [27] and a cosmetic ingredient in moisturizing and protective skin treatment cosmetic creams [24,27,28].

Applications in Food industry: Alginates are added as organoleptic additives and stabilizers in food to improve, modify and stabilize the texture of foods. It possesses properties such as viscosity enhancement, gel forming ability, stabilization of aqueous mixtures, dispersions and emulsions. It can be used to prepare restructured food based on its ability to form gels of calcium alginate which is independent of temperature. Examples of such restructured foods include pimento olive fillings, cocktail berries, crab sticks, onion rings, meat products for humans and pets etc. It can also be used as a coating for citrus fruits, an inert pesticide adjuvant, and as a component in the preparation of paper and paper board in contact with fatty and aqueous foods [29].

MATERIAL AND METHODS:

Bacterial cultures : Azotobacter vinelandii (AV); Azotobacter chroococcum (AC); Pseudomonas species (PS); Unidentified (UN).

Medium for cultivation : The cultures were grown in Burk's medium at 30^{o}C for 5 days, supernatant was withdrawn after 5 days by carrying out centrifugation at 8000 rpm for 20 minutes. Crude supernatant was directly used for assay by Carbazole, Blumenkrantz and Asboe-Hansen method. Further the polysaccharide was extracted and precipitated from the supernatant

as described by [30,31] (Van der Berg et al, 1995; Hacking et al, 1983) and analyzed spectophotometrically. Hydrolysis of extracted polysaccharide was carried out by the method of Chandia et al.,2001 [32]; separated fractions were used for carrying out TLC.

Growth conditions : The cultures were grown in Burk's medium at 30°C with 5% inoculum level and incubated for 5 days at 100 rpm.

Carbazole method: 0.1 % of carbazole is dissolved in ethanol, Conc.H_2SO_4, borate stock, H_2SO_4 borate reagent (Preparation: 24.74 gm H3BO3 was dissolved in 45 ml of 4 M KOH. The solution was diluted to 100 ml with double distilled water so as to get the 4M solution. The sensitivity of the method is upto 1000 µg/ml. The reaction is performed in two sets:

Reaction without borate at 55°C : 1 ml of alginate solution was kept in ice-bath for 10 min for equilibration. Then 6 ml of chilled conc. H_2SO_4 was added. Further it was kept in ice-bath for re-equilibration. Mixed by vortexing and heated at 55°C for 20 minutes in a water bath. Re-equilibration was done in ice-bath for 10 minutes with subsequent addition of 0.2 ml of carbazole reagent. Sample was mixed with a vortex mixer and allowed to incubate at room temperature for 3 hours for colour development. Blank was prepared using double distilled water instead of sample. Absorbance was read at 530 nm.

Reaction with borate at 55°C : 6 ml of H_2SO_4 –borate reagent was added in a test-tube and kept for 10 min in ice-bath for equilibration. Extracted polysaccharide or alginate solution (0.7 ml) and kept in ice-bath for 10 min. Mixture was agitated using vortex mixer for 4 seconds and then re-equilibrated in ice-bath for 10 minutes. 0.2 ml of Carbazole solution was added, mixed and heated at 55°C for 30 minutes. Blank was prepared using double distilled water instead of sample. Reaction mixtures were cooled and absorbance was read at 530 nm. Concentration was determined by comparing the results with the standard graph of glucuronic acid. Further M:G ratio was determined with the formula [33] given by Knutson & Jeans 1968.

$$R = \frac{A'_{mix}}{A_{mix}} = \frac{f_1 A'_1 + (1-f_1) A'_2}{f_1 A_1 + (1-f_1) A_2}$$

Blumenkrantz and Asboe-Hansen method: This method is rapid but is accurate only upto 150 µg/ml. Broth cultures were used for this assay as the medium supernatant used does not interfere with the color development. Supernatent was treated with 3% TCA to precipitate the proteins

[34]. Reagent preparations - 12.5 mM sodium tetraborate in concentrated sulfuric acid. 0.15% (w/v) meta-hydroxydiphenyl in 0.5% (w/v) sodium hydroxide. 0.2 ml of sample was taken in a test tube and 1.2 ml of sodium tetraborate was added. Blank was prepared using plain medium. The mixture was shaken in a vortex mixer and the tubes were heated in water bath (100°C) for 5 min. Cooled in ice-bath and 20 ml of m-hydroxydiphenyl reagent was added. Tubes were shaken thoroughly and the absorbance was read at 520 nm. The value was compared with the standard graph of glucuronic acid to obtain the concentration of uronic acid.

UV-visible spectrophotometric analysis: Broth cultures were centrifuged at 8000 rpm for 20 minutes to remove the cell pellet. Supernatant was treated with 3% chilled TCA and was kept under cold conditions for protein precipitation. The sample was centrifuged at 8000 rpm for precipitating the proteins. Supernatant was used to carry out spectral studies in the range of 200 – 300 nm. Absorption of various samples was noted at 213 nm [35].

Confirmation by TLC:

Partial hydrolysis of polysaccharide sample : Partial hydrolysis of the extracted polysaccharide was carried out by the modified method of Chandia et al., 2001 [32]. Three grams of the sample was dissolved in double distilled water, then 9 ml of 3 M HCl was added and refluxed for 20 min. The suspension was cooled down and further centrifuged at 10,000 rpm for 20 min. The obtained precipitate was dissolved in 300 ml of 0.3 M HCl and refluxed further for 2 hours. The solution was cooled and centrifuged at 10,000 rpm for 20 min, 1 M NaOH was used to neutralize the precipitate. 1 M HCl was used to adjust the pH to 2.85 ± 0.5. Centrifugation was carried out at 10,000 rpm for 15 min, the insoluble polyguluronate fraction and the soluble mannuronate fraction was collected and neutralized with 1 M NaOH. pH of both the fractions were adjusted to 2.85 ± 0.5 with 1 M HCl. Ethanol was used for precipitation and washing of the samples, the samples were further dried.

TLC aluminium plates of silica gel 60 were used to separate out the hydrolyzed samples.

Sample preparation : 2.5 mg of sample in 500 µl of double distilled water. 5µl of samples were spotted on the plates, modified solvent system n-butanol/ acetic acid/water (3:2:2, v/v/v) was used. The sample was run by ascending chromatographic technique. Locating reagent: 10%

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sulfuric acid in ethanol. Plates were sprayed with locating reagent and heated at 110°C for 5 min [23]. The results were compared with the standard glucuronic acid.

RESULT AND DISCUSSION :

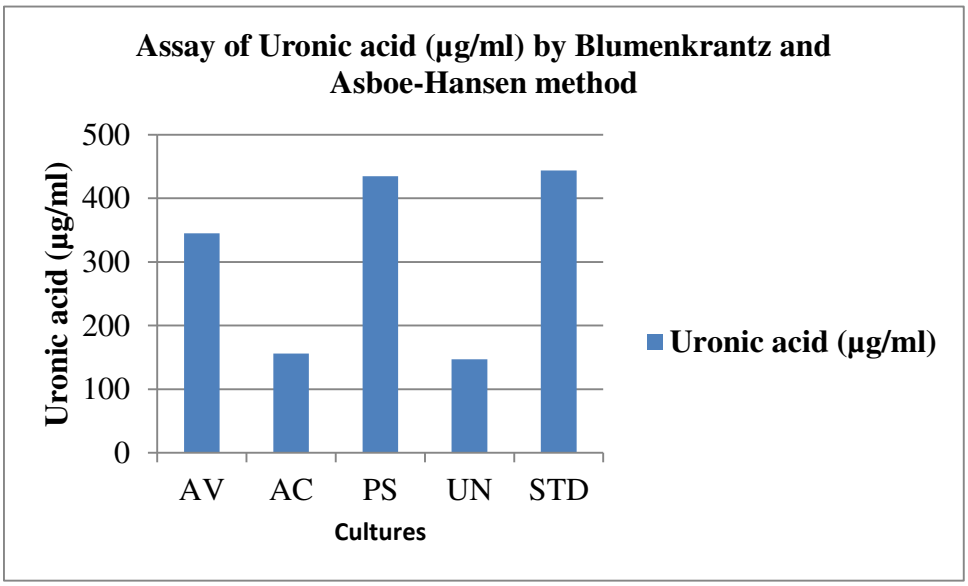

Figure 1: Determination of uronic acid content by Blumenkrantz&Asboe-Hansen method.

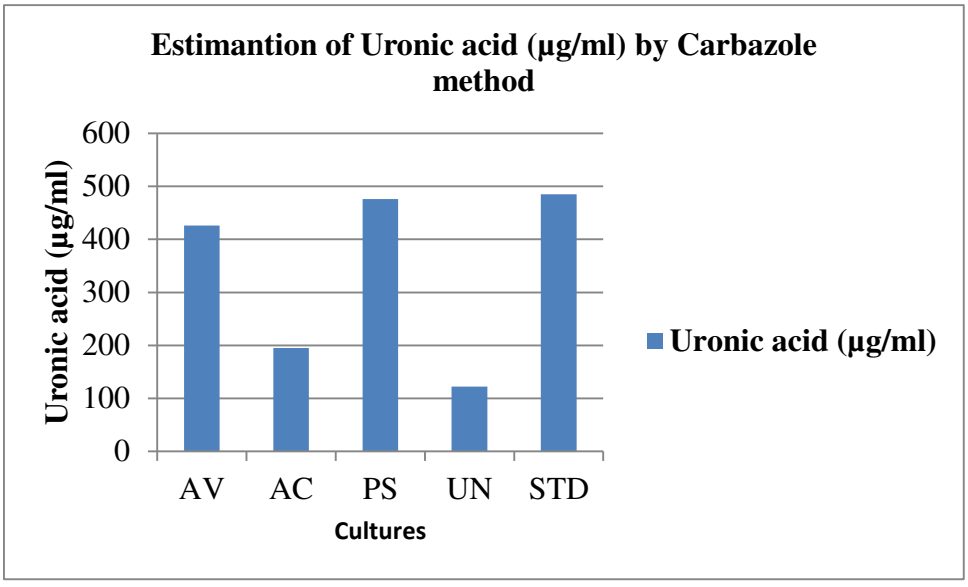

Figure 2: Determination of uronic acid by Carbazole test.

As evident from fig.1 and fig.2 uronic acid concentration was found to be more in AV & PS as compared to AC and UN by both the methods (Blumenkrantz and Asboe-Hansen, Carbazole).

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The uronic acid content is in the range of 350 – 500 µg/ml as calculated from the standard readings of glucuronic acid.

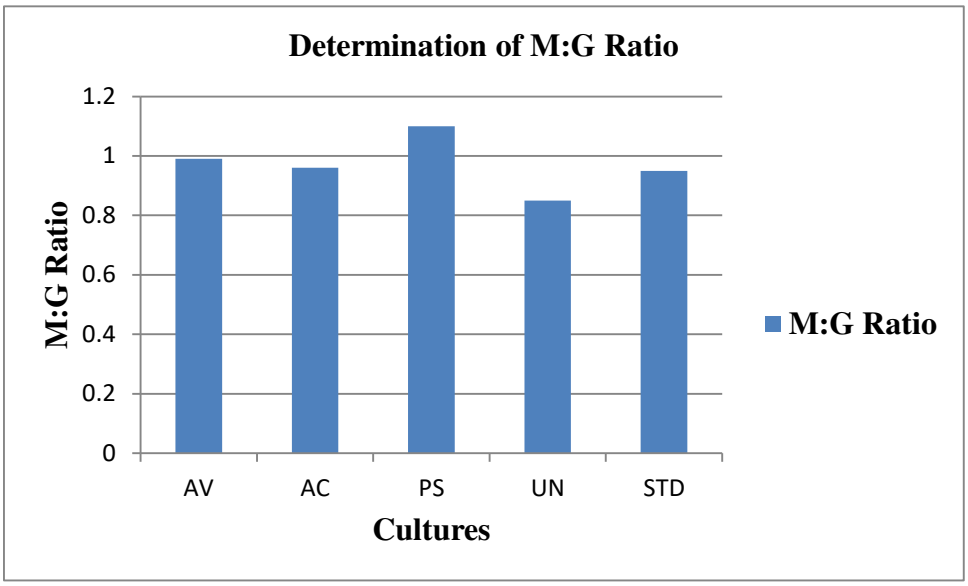

Figure 3: Determination of M:G ratio.

M:G ratio was found to be varying among all. From fig.2 its clear that PS is having more mannuronic content and AV is having high guluronic content.

Table 1: Spectrophotometric analysis.

Sample	Optical density at 213 nm
AV	1.817
AC	1.016
PS	1.723
UN	1.009
Alginate (Std.)	1.923

Table 1 indicates maximum polysaccharide production in liquid broth after 5 days of incubation for cultures AV and PS.

Table 2: TLC of extracted samples

Sample	Rf value (M)	Rf value (G)
AV	0.38	0.33
AC	0.38	0.32

PS	0.37	0.34
UN	-----	-----
Alginate (Std.)	0.39	0.32
Glucuronic acid (Std.)	0.42	

Table 2 shows the Rf value of separated monomers which is nearby the standard Rf value thus showing the presence of a uronic acid monomer in the extracted polysaccharide sample from different sources. Knutson and Jeanes (1968) [33] found carbazole analysis at 55^{o}C to be the best method to reduce interference by neutral sugars. Blumenkrantz & Asboe-Hansen replaced carbazole with m-hydroydiphenyl. Reactions were carried out at ambient temperatures to avoid second heating that gives substantial interference by neutral sugars. Tulia & Nicolas (1991) found that Blumenkrantz & Asboe-Hansen is quite suitable for pectins and other polymers containing major amount of uronic acids. If uronic acid is in low concentration neutral sugars interfere more by browning on heating. Addition of sulfamate minimizes this effect to a greater extent [36]. The results are consistent with those obtained by Vineet Kumar et al., 2014 who used both Blumenkrantz and Asboe-Hansen & Carbazole methods for estimation of uronic acids [37]. TLC technique was employed for the detection of acid hydrolyzed product of alginate [10,38,39,40]. UV-vis spectrophotometric analysis of the enzymatically hydrolyzed products of alginate has been performed by various researchers [41,42,43,44,45]. Maximum absorption at 213 nm in UV-vis spectrophotometer is attributed to the presence of hydroxyl and carboxyl groups [46,35].

CONCLUSION :

Uronic acid containing polysaccharides has widespread applications. The intact or hydrolyzed products are used for various purposes which also includes food industry. Preliminary methods discussed in this paper are easy to perform and helps in rapid detection of uronic acids or identifying such polysaccharides in the early stage of research work.

DECLARATION :

Authors declare no conflict of interest.

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