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Fibrinolytic, anti-inflammatory and anti-microbial properties of α-(1-3)-glucans produced from *Streptococcus mutans* (MTCC 497)

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ABSTRACT

Streptococcus mutans (MTCC 497) cell associated α-(1-3)-glucans were isolated, characterized and evaluated for their bioactivity profile. Acid hydrolysis of α-(1-3)-glucans revealed presence of glucose moieties. Water insoluble α-(1-3)-glucans (WIG) were sulfated to convert them into water soluble glucans which were characterized by FT-IR spectral studies. The sulfation of WIG was confirmed by the presence of —O—SO_3— and C—O—SO_3— characteristic peaks at 1240 and 820 cm^{-1}. MALDI-TOF analysis of sulfated α-(1-3)-glucan revealed 1.2 to 9 kDa fragmentation. Antibacterial profile studies revealed higher growth inhibitory activity against Gram negative than Gram positive bacterial strains by sulfated α-(1-3)-glucans. One-fold higher anti-inflammatory activity with IC_{50} value of 0.11 mg/ml was observed with sulfated α-(1-3)-glucans over WIG. Time dependent fibrinolytic potential without requirement of tissue plasminogen activators was observed for sulfated α-(1-3)-glucans. This is the first report demonstrating the fibrinolytic and anti-inflammatory property for sulfated α-(1-3)-glucans.

1. Introduction

Glucans are polysaccharides of repeating units of either mono- or disaccharides joined together by glycosidic bond. These are often quite heterogeneous, non-allergenic, water insoluble cell wall components of fungi, certain bacteria and plants (Williams, 1997) and differ in repeating unit and also at bonding pattern alpha (α) or beta (β) with variety of branching positions (1-2, 1-3, 1-4 and 1-6). Depending on the structure and bonding pattern, these macromolecules acquire distinct properties from their monomers and acquired significance in health sector as nutraceuticals and play beneficial role in therapeutics especially in treatment of cancer, immunodeficiency, or indiscriminate immunosuppression associated drug treatment, in combinational therapy with antibiotics and as adjuncts for vaccines (Jong & Birmingham, 1992). Reports are also available on glucans mediated momentous assurance in the healing of contagious diseases (Muller et al., 1997). Chemical synthesis of these glucan is a tedious, energy-intensive and laborious process. Hence, world-wide scientific community is searching for glucan production through biological process.

Glucan polysaccharides are anabolic products synthesized by an array of diverse microbial genera including *Streptococcus* sps. (hyaluronic acid: glucan polymer with β-1,4 and β-1,3 bonding) (Schrager, Alberti, Cywes, Dougherty, & Wessels, 1998), *Bacteriodes fragilis* (polysaccharide A) (Shapiro et al., 1986), *Lentinus edodes* (α-glucans: glucan with α-(1-3) bonding) (Surenjav, Xiaojuan, Fanbo, & Lina, 2006), and *Saccharomyces cerevisiae* (β-glucans with β-1,3 and β-1,6 bonding) (Muller et al., 1997) that produce either intracellular or extracellular or associated with cell walls as an integral part of cell membrane. Out of two glucan polysaccharides, β-glucans are comprehensively studied mainly due to their abundance in fungal cell wall components up to 60% of the dry weight (Klis, 1994) and has been used as ancestral medicine in China and Japan (Lindequist, Niedermeyer, & Julich, 2005; Mayell, 2001). Information from scientific communities also suggests that several fungal β-glucans may be emerging as effective immunomodulatory, and may impact positively on cancers and several bacterial infections (Brown & Gordon, 2001; Vetvicka & Yvin, 2004). In Japan lentinan (β-glucans with β-1,3 and β-1,6 bonding) was accepted for clinical treatment of gastric and colorectal cancers, and encouraging survival rates have been reported (Munemoto et al., 2002; Nakano et al., 1999).

Compared with the intensive investigations on β-glucans, sparse reports are available on α-(1-3)-glucans and their pharmaceutical applications mainly due to their rare natural occurrence

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and water insoluble nature. However, their presence in several microbial strains as a constituent of cell membrane has been reported albeit at low concentrations compared their counterparts *i.e.*, β-glucans. Zhang, Zhang, and Cheng (2002) reported that α-(1-3)-glucans isolated from fungal cell wall revealed the anti-cancer activity against epithelial sarcoma and breast cancer cell lines. In another study, the same group also reported immuno-modulating properties of α-(1-3)-glucans isolated from *Lentinus edodes* cell walls (Zhang et al., 2002). Though some reports are available on the production of α-(1-3)-glucans by *Lactobacillus* group organisms (*Lactobacillus mesenteriodes*), limited information is available on their utility spectra. In the present study, an effort has been made to produce water insoluble α-(1-3)-glucans (WIG) directly from *Streptococcus mutans* (MTCC 497) and characterize for their bonding pattern in addition to evaluation of their potential for fibrinolytic, anti-inflammatory and anti-microbial properties. To the best of our knowledge, this is the first report on *in vitro* antimicrobial, fibrinolytic and anti-inflammatory properties of α-(1-3)-glucans from bacterial or fungal source.

2. Materials and methods

The cultures *Escherichia coli* MTCC 1680, *Bacillus sphaericus* MTCC 7542 and *Staphylococcus aureus* MTCC 7405 are obtained from Andhra University, Visakhapatnam. *Pseudomonas aeruginosa* MTCC 6458, *Bacillus cereus* MTCC 9762 and *S. mutans* MTCC 497 were procured from Microbial Type Culture Collection, IMTECH, Chandigarh. *Stenotrophomonas maltophilia* (HE963840) was isolated in our laboratory. All chemicals and dehydrated culture media were procured from HIMEDIA, India while solvents used in this study were purchased from SD Fine Chemicals Ltd., India.

2.1. Culture revive

Lyophilized culture of *S. mutans* (MTCC 497) was obtained from IMTECH (Institute of Microbial Technology), Chandigarh. The obtained culture was revived using brain heart infusion (BHI) medium at 32 °C for 48 h. Fresh cultures were maintained on BHI agar slants and stored at 4 °C until further use.

2.2. Production and extraction of water insoluble glucans

Active culture of *S. mutans* (MTCC 497) was utilized for the production of water insoluble glucans (WIG). Initially, *S. mutans* (MTCC 497) was grown in 25 ml of BHI medium for inoculum preparation for 18 h at 32 °C under aerobic environment. The production of biomass was carried out at 150 rpm and at pH 7.2 by inoculating 2.5% (v/v) (1 ml = 1×10^8 CFU) of inoculum under sterile conditions in a BHI medium supplemented with 50 g/L sucrose. After 72 h of incubation, the produced biomass was separated by centrifugation at 15,000 rpm for 15 min. The obtained pellet was used for extraction of WIG. In brief, for extraction of WIG, initially cell pellets were washed with sterile deionized water and subsequently the cell pellet was treated with 150 ml of 3 N KOH at 95 °C. After 2 h the samples were cooled to room temperature and centrifuged at 15,000 rpm for 30 min. The obtained supernatant was neutralized with glacial acetic acid and the samples were stored at 4 °C overnight for the precipitation of glucans. The precipitated glucans were separated by centrifugation and lyophilized to obtain glucan powder. The lyophilized glucan powder was utilized for further studies.

2.3. Sulfation of glucans

Sulfation of WIG was performed according to Zhang et al. (2002). Briefly, a known amount of (200 mg) of α-(1-3)-glucan was dissolved in 50 ml of DMSO supplemented with 0.25 M LiCl. The mixture was homogenized for 4 h at 80 °C on magnetic stirrer at 400 rpm for proper distribution of glucans. One gram of sulfur trioxide–pyridine complex was added to the above reaction mixture and sulfation reaction was continued. After 72 h the reaction mixture was cooled to room temperature and then adjusted the pH of reaction mixture to 7.5 by using 0.2 N NaOH. Sulfated glucans were separated by centrifugation at 15,000 rpm for 30 min and the pellet was lyophilized to powder for further analysis.

2.4. FTIR characterization of water insoluble and sulfated glucan

For Fourier transform infra-red (FT-IR) spectroscopy measurements, 5 mg of freeze dried WIG and sulfated α-(1-3)-glucans along with potassium bromide were taken separately in the ratio of 1:100. FT-IR spectrum of samples was recorded on FT-IR instrument with diffuse reflectance mode (DRS-800) attachment. All measurements were carried in the range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}.

2.5. Test of homogeneity and complete acid hydrolysis

A 30 mg of WIG was pretreated with 0.3 ml of 72% H_2SO_4 for 1 h at 37 °C. Then 8.4 ml of deionized water was added to bring acid concentration to 4%. This solution was boiled at 121 °C for 60 min at 15 lb pressure. The solution was rapidly cooled, neutralized with calcium hydroxide ($Ca(OH)_2$). The resultant hydrolysate was subjected to HPLC analysis and Osazone test for the confirmation of monosaccharide. Monosaccharide were determined using a Waters Alliance HPLC (Waters, Japan) system fitted with a phenomenex H^+ (monosaccharide) column and refractive index detector, by injecting 20 μL sample using following conditions. Mobile phase 5 mM H_2SO_4 (flow rate: 0.5 ml/min), oven temperature 60 °C was used. Standard glucose, xylose, maltose, fructose and galactose were used for comparison.

2.6. MALDI-TOF studies for WIG and sulfated glucans

MALDI-TOF MS experiments were performed on a Shimadzu biotech AXIMA performance MALDI-TOF/TOF (Shimadzu Corporation, Japan). Positive ion measurements were taken in reflection mode using a UV laser (337 nm) with an acceleration voltage of 20 kV and a delayed ion extraction setting of 20 ns. The samples of WIG and sulfated α-(1-3)-glucans homogenized in methanol with a fixed amount of matrix of *p*-chloro-α-cyanocinnamic acid (ClCCA) were loaded by a dried droplet method. Typically, spectra were obtained by accumulating 300 laser shots for quantification.

2.7. Anti-microbial properties

Biosynthesized WIG from *S. mutans* (MTTC 497) and chemically converted sulfated α-(1-3)-glucans were tested for antimicrobial activity by using three Gram-negative (*E. coli*, *S. maltophilia* and *P. aeruginosa*) and three Gram-positive (*S. aureus, B. sphaericus* and *B. cereus*) by the well-diffusion method. In brief, approximately 20 ml of nutrient agar medium was poured into sterilized Petri dishes. The bacterial test organisms were grown in a nutrient broth for 24 h. A 50 μl nutrient broth culture of each bacterial organism (1×10^5 CFU/ml) was used to prepare bacterial lawns. Agar wells of 5 mm diameter were prepared with the help of a sterilized stainless steel cork borer. The wells were loaded with 30 μl of 1 mg/ml of WIG and 30 μl of 1 mg/ml of sulfated α-(1-3)-glucans, along with 30 μl of 100 μg/ml of streptomycin as a standard (a positive control) or dimethylsulfoxide (DMSO) as negative control. The plates were incubated at 37 °C for 24 h and examined for the presence of zones of inhibition. The diameter of zones of inhibition was measured and the mean value for each organism was recorded and expressed

in millimeter unit. Minimum inhibitory concentration (MIC) was determined by the broth dilution method and Minimum bactericidal concentration (MBC) was determined by the plate method.

2.8. In vitro anti-inflammatory activity

The anti-inflammatory activity of WIG and sulfated α-(1-3)-glucans was studied according to Leelaprakash and Mohan (2011) by using inhibition of albumin denaturation technique. The reaction mixture (pH 7.0) consists of test extracts and 1% aqueous solution of bovine albumin. Reaction mixture were incubated at 37 °C for 20 min and then heated to 70 °C for 20 min. After cooling the samples to room temperature, the turbidity was measured at 660 nm using UV–visible Spectrophotometer (Model SL 210, Elico India Ltd.). The experiment was performed in triplicate and average values were reported. The percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition = [(Abs control − Abs sample)/Abs control] × 100

2.9. Fibrinolytic activity

Fibrinolytic activity was determined using the method described by Lakshmi and Prakasham (2013) with following modifications. In brief, the fibrin clots were made in eppendrof tubes by cross-linking of fibrinogen (1 ml of 10 mg/ml human fibrinogen) in the presence of 0.1 ml of 52 units/ml human thrombin (Sigma-Aldrich). All solutions were made using 20 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl buffer. The developed clot was allowed to stand for 1 h at 37 °C in incubator. Then, 10 μl of test (WIG and sulfated α-(1-3)-glucans) solution was added to each tube except one tube which served as control. The tubes were incubated at 37 °C and fibrin lysis was observed.

3. Results and discussion

3.1. Production and extraction of water insoluble glucans

In the present study, WIG was produced from stationary phase cells of *S. mutans* (MTCC 497) which were grown in brain hart infusion medium using sucrose as carbon source at 37 °C at pH 7.0 by incubating at 150 rpm. Systematic evaluation of WIG production during fermentation revealed that WIG production increased with increase of cell mass suggesting WIG production is growth associated and is constitutive in nature. Similar trend has been observed by Adrian, Adam, and Janusz (1999) and Ebisu, Kato, Kotani, and Misaki (1975) by *S. mutans* 20381 and *S. mutans* OMZ 176, respectively, however, α-(1-3)-glucans production values varied. A maximum of 1.27 g of α-(1-3)-glucan was noticed in 72 h in the present study while approximately three-fold reduced yield was observed by Adrian et al. (1999) where authors reported 317 mg α-(1-3)-glucans under similar fermentation conditions by using *S. mutans* 20381. Whereas, Ebisu et al. (1975) reported much lower WIG production (199 mg) by the *S. mutans* OMZ176. Such variations in WIG production by *S. mutans* (MTCC 497) under similar fermentation conditions and sucrose as carbon source noticed in the present study and by Adrian et al. (1999) along with Ebisu et al. (1975) may be attributed to the seriotypic properties of *S. mutans*. The observed higher productivity of WIG by *S. mutans* (MTCC 497) has significant industrial implications (Masakazu & Toshihiko, 1979). This is because WIG play a pivotal role in health and therapeutic benefits (Manisha, Rohit, & Shubhini, 2010; Surenjav et al., 2006) and it is well established that WIG from fungal and mushroom sources are best examples for nutraceuticals (Jong & Birmingham, 1992) though none was reported from bacterial sources.

Table 1
Elemental analysis of glucan and sulfated glucan.

S. no.	Element (%)	Glucan	Sulfated glucan
(1)	Carbon	35.55	35.53
(2)	Nitrogen	0.00	0.00
(3)	Hydrogen	6.682	3.357
(4)	Sulfur	0.00	13.468

3.2. Sulfation of glucans

Nutraceutical application potential of α-(1-3)-glucans depends on its eukaryotic cell metabolic interaction. Any product metabolic interaction mainly depends on the product properties especially polarity which mainly help in transport of product to metabolic site from external medium environment. In view of low polarity of WIG and higher molecular weight (ranges from 50 to 300 kDa), their interactive nature with cellular metabolic pathway is restricted even at transport from external to intracellular level. Furthermore it was noticed that water solubility of the sulfated α-(1-3)-glucans were greatly increased. Up on sulfation, the degree of substitution (DS) was noticed to be 1.192 and yield of the product obtained was 187 mg corresponding to 93% after sulfation. This data is in accordance with the reported percentage of sulfated glucans isolated from fungal sources by Chen, Yu, and De (2013) and Zhang et al. (2002) where only 85–90% yield was reported. The sulfur molecule presence on sulfated glucans was also further confirmed by FT-IR spectroscopy and elemental analysis (Table 1).

3.3. FT-IR characterization

Even though polymers are complicated substances for analysis by FT-IR spectroscopy and it is widely employed in solving many problems involving their structures. FT-IR spectra for WIG and sulfated α-(1-3)-glucan were shown in the Fig. 1. The occurrence of characteristic stretching vibration at 1634 cm^{-1} in both WIG and sulfated α-(1-3)-glucans revealed that these two compounds are polysaccharides in nature. Similar vibrations were reported by Surenjav et al. (2006) however for β-glucans. Presence of stretching vibrations at 3400 cm^{-1} and at 2924 cm^{-1} in both WIG and sulfated α-(1-3)-glucans reveal the existence of OH and CH groups (CH_3 or CH_2) on the polymer with sugar backbone (Chen et al., 2013). The presence of peak at 924 cm^{-1} for WIG and 953 cm^{-1} for sulfated α-(1-3)-glucans suggested that both of these glucans posses α-(1,3)-bond which corresponds to C—O—C glycosidic linkage of polysaccharide. The absence of any other glycosidic linkage peak at 1160 cm^{-1} indicated that the produced glucan was linear in structure according to Venkanna, Adhikary, and Krishnamoorthi (2012). Further, FT-IR spectrum of sulfated α-(1-3)-glucans showed the characteristic absorption peaks of S=O and C—O—S stretching vibrations at 1240.21 cm^{-1} and 820.36 cm^{-1}, respectively, indicating that sulfates were introduced into the sugar moieties of WIG. The presence of peak at 820 cm^{-1} was tentatively attributed to 6-O-sulphate group according to the report published by Grant, Long, Moffat, and Williamson (1991) suggesting that the sulfation was occurred at the hydroxyl group attached at C6 carbon.

3.4. Acid hydrolysis studies

Complete acid hydrolysis of WIG, produced in the presence of sucrose by *S. mutans* (MTCC 497), up on reaction with phenyl hydrazine showed needle shaped crystals under microscopic

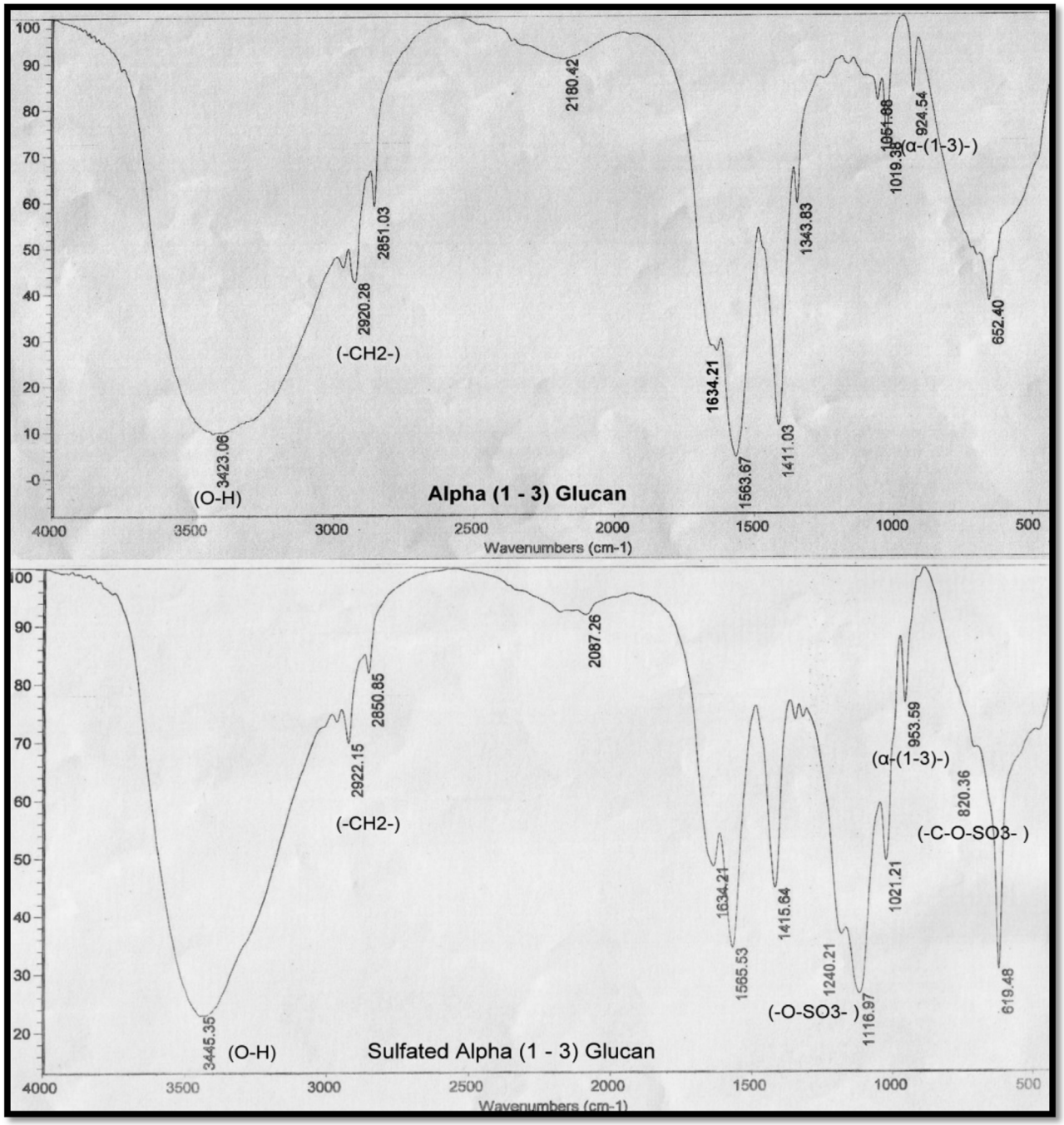

Fig. 1. FTIR spectrum for glucan and sulfated glucan.

observation (Fig. 2). The needle shaped crystals do suggest that WIG is a polymer of either glucose or fructose units. To resolve further to know whether the monomeric unit of WIG is glucose or fructose, the time required for needle formation is considered. According to Sherman and Williams (1906) glucose mediated needle shaped crystals known to be formed within 4 min of phenyl hydrazine interaction while fructose mediated crystals will be observed only within one minute of interaction. Since, in the present study, needle formation was noticed after 4 min of phenyl hydrazine interaction, it was concluded that the WIG produced is a polymer of glucose monomers which was further confirmed by HPLC analysis. This was the first report on Osazone formation for the acid hydrolysis of water insoluble glucans. HPLC analysis of complete acid hydrolyzed product revealed the presence of single major peak at a retention time 13.23 min corresponding standard glucose indicating the produced glucan is synthesized by glucose monomers.

3.5. MALDI-TOF studies for sulfated glucans

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) was used for analysis of sulfated α-(1-3)-glucans as this method was effectively developed as a soft ionization method in MS revealing for biopolymers and macromolecules (Marvin, Roberts, & Fay, 2003). This is because most polysaccharides have colossal molecular weights and show meager ionization efficiency, almost certainly due to their underprivileged capability in carrying charge for mass spectrometric detection. It was noticed from the Fig. 3, that the molecular weight of the sulfated α-(1-3)-glucans

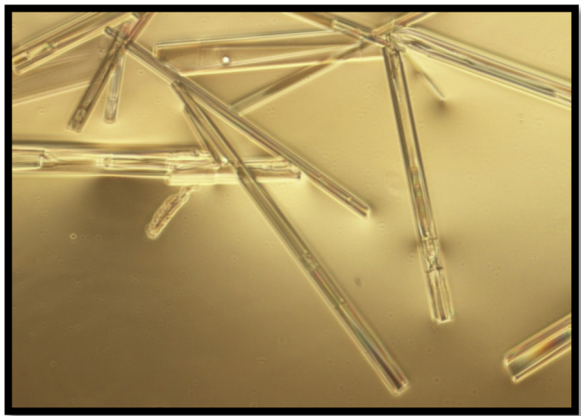

Fig. 2. Needle shaped glucosazone formation after treating the acid hydrolysate with phenyl hydrazine.

were in a range from 1.2 to 9.0 kDa which were very low when compared to native α-glucans (data not shown). The observed lower molecular weight of sulfated α-(1-3)-glucans was attributed to degradation of native glucans during sulfation at high temperature (80 °C) similar to that noticed by Zhang et al. (2002). Similar study was reported by Pingyi and Peter (2002) where 57% (16.1×10^4) of reduction in molecular weight when compared to native glucan molecular weight.

3.6. Antimicrobial activity

In order to evaluate antimicrobial role of glucan polymers, anti-bacterial activity of biosynthesized WIG and sulfated α-(1-3)-glucans was investigated against various pathogenic bacteria such as *E. coli, S. aureus*, *B. sphaericus*, *S. maltophilia*, *P. aeruginosa* and *B. cereus*. Table 2 indicated the antibacterial activity profile of WIG and sulfated α-(1-3)-glucans against negative control (DMSO) along with streptomycin sulphate as positive control. Biosynthesized WIG did not show any antibacterial activity whereas, sulfated α-(1-3)-glucans exhibited antibacterial activity against all tested microbial strains (Fig. 4). The observed difference in antibacterial activity between WIG and sulfated α-(1-3)-glucans may be attributed to solubility properties of both these glucans. The enhanced solubility of sulfated α-(1-3)-glucans may be attributed to substitution of hydroxyl groups with sulfur trioxide groups along with its low molecular weight compared to WIG (Fig. 3). Critical

Table 2
Antibacterial activity of glucan and sulfated glucan against pathogenic bacteria.

Organism	Zone of growth inhibition in mm		
	WIG[a]	Sulfated glucan	Streptomycin
Pseudomonas aeruginosa	00	25 ± 02	23 ± 03
Stenotrophomonas maltophilia	00	21 ± 01	16 ± 02
Bacillus sphaericus	00	14 ± 02	34 ± 02
Bacillus cereus	00	00	22 ± 01
Escherichia coli	00	28 ± 01	30 ± 01
Staphylococcus aureus	00	16 ± 02	27 ± 02

[a] Water insoluble glucans.

evaluation of antibacterial properties of sulfated α-(1-3)-glucans further suggested that 30% higher inhibitory role of sulfated α-(1-3)-glucans was noticed against *S. maltophilia* while almost equal inhibitory activity against *P. aeruginosa* whereas lower inhibitory activity against *B. sphaericus* and *B. cereus* compared to positive control *i.e.*, streptomycin. Further analysis of this antibacterial profile of sulfated α-(1-3)-glucans with respect to nature of bacterial cell wall suggested that gram negative bacterial strains are more susceptible to that of gram positive. This conclusion may be evidenced from the fact that higher or similar antibacterial activity of sulfated α-(1-3)-glucans and streptomycin was noticed with *P. aeruginosa, S. maltophilia* (belongs to gram negative) while the tested gram positive strains, *B. sphaericus and B. cereus*, revealed higher resistance compared to positive control (streptomycin). This variation of antibacterial profile of sulfated α-(1-3)-glucans may be due to the diffusion property as gram negative bacterium contain less percentage of peptidoglycan layer (a major component of bacterial cell wall) to that of gram positive bacterium (Gram positive bacterial cell wall contain 70% peptidoglycans while Gram negative contain only 30%). In view of observed 30% higher antibacterial activity of sulfated α-(1-3)-glucans against *S. maltophilia*, an opportunistic pathogen, use of sulfated α-(1-3)-glucans may be evaluated further for treating diseases associated with *S. maltophilia* such as bloodstream, respiratory tract and urinary tract infections. Working with glucan mediated antimicrobial activity, Shin, Lee, Lee, and Lee (2005) and Di, Williams, McNamee, Edwards, and Kitahama (1979) reported antibacterial activity for aminated glucans and native glucans against *E. coli* and *B. subtilis*, however, aminated glucans showed more than one folds higher activity to that of native glucans. The major difference between Shin et al. (2005) and Di et al. (1979) to that of present study is that the former reports used water soluble β-glucans while currently studied α-(1-3)-glucans

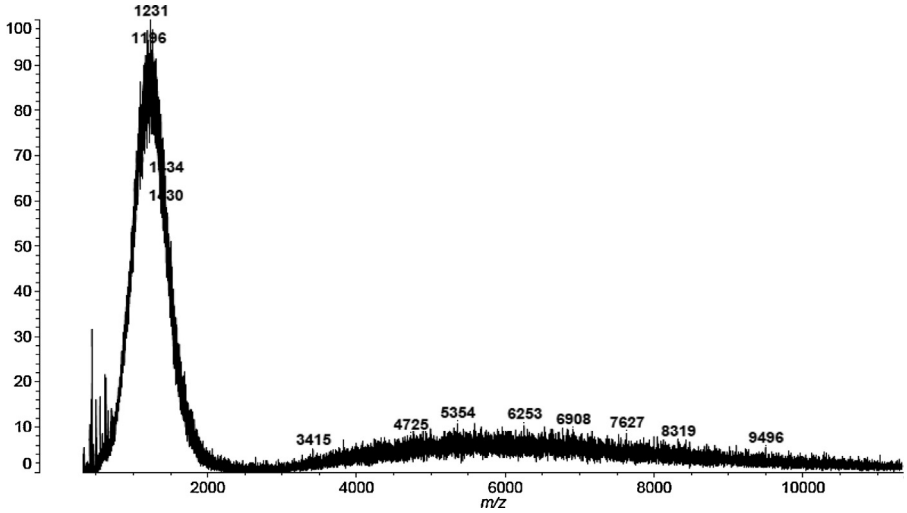

Fig. 3. MALDI-TOF spectrum of sulfated alpha glucans.

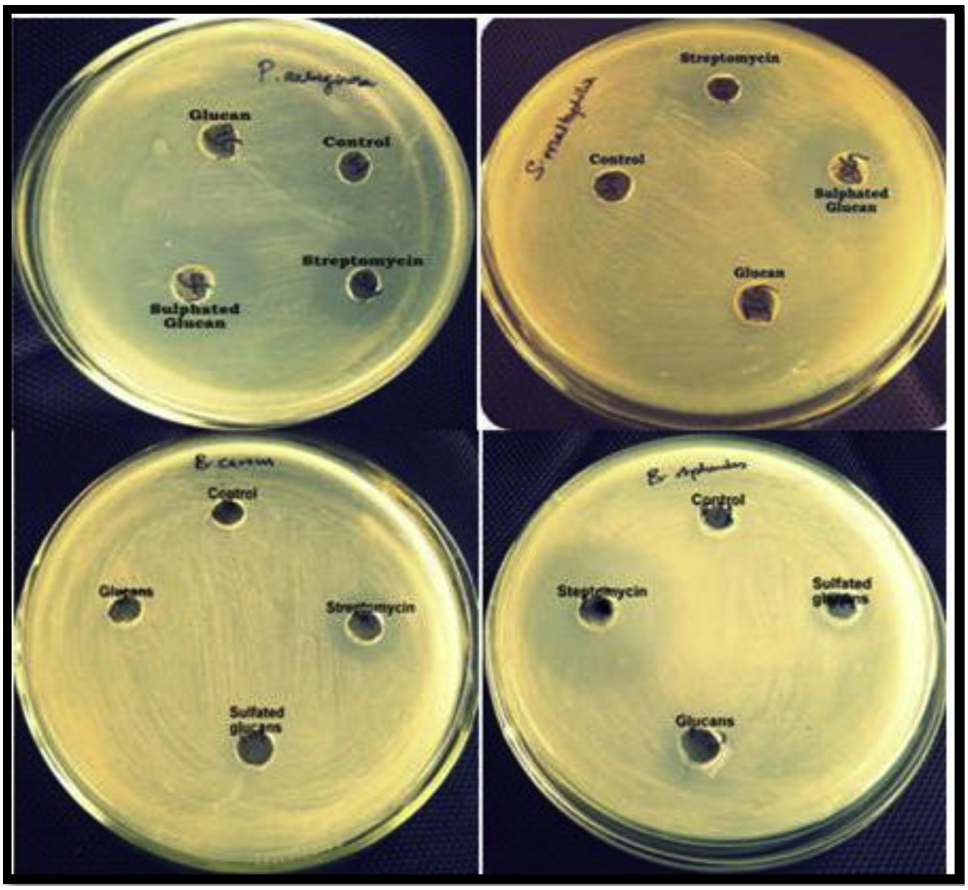

Fig. 4. Antimicrobial activity of glucans and sulfated glucans.

are water insoluble in their native forms. This could be major difference for observed antibacterial profile of α-(1-3)-glucans *vis* a *vis* β-glucans reported by Shin et al. (2005) and Di et al. (1979). To understand further, MIC/MBC properties of WIG and sulfated α-(1-3)-glucans were evaluated. It was observed that only sulfated α-(1-3)-glucans showed MIC/MBC properties which may be attributed to solubility nature. Among the tested bacterial strains, *E. coli* exhibited a lowest MIC/MBC value (50 μg/ml) whereas *B. cereus* and *B. subtilis* showed a highest MIC/MBC value (200 μg/ml) while *P. aeruginosa*, *S. maltophilia* and *S. aureus* exhibited a MIC of 100 μg/ml and MBC of 200 μg/ml. The observed little variation in MIC/MBC values further suggest that the sulfated α-(1-3)-glucans has bactericidal property. The data also confirm that MIC and MBC values for sulfated α-(1-3)-glucans differ with genetic nature of bacterial strains.

3.7. *In vitro anti-inflammatory activity*

Most biological proteins lose their natural function when denatured leading to inflammation. During denaturation of proteins they lose their complex tertiary structure by application of external stress or compound, which may be a strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Compounds which inhibit the denaturation or increase the stability of proteins may term as anti-inflammatory. The imperative role of α-(1-3)-glucans (with and without sulfation) as anti-inflammatory agents was investigated against the heat mediated protein denaturation and compared with the diclofinac sodium as standard anti-inflammatory drug. The data presented in Table 3 revealed that both sulfated α-(1-3)-glucans and WIG revealed the anti-inflammatory property, however, a variation was noticed between them on the percent activity. More pronounced anti-inflammatory property has been observed against sulfated α-(1-3)-glucans. This can be evidenced from observations that (a) use of 100 μg/ml concentration of WIG and sulfated α-(1-3)-glucans revealed anti-inflammatory activity of 37 and 45%, respectively and (b) increase of sulfated α-(1-3)-glucan concentration in the reaction medium from 100 to 500 μg/ml resulted in significant improvement of anti-inflammatory activity from 45% to 82% while use of WIG showed little improvement in anti-inflammatory activity against the same concentration gradient (Table 3). This observed variation of anti-inflammatory property of sulfated α-(1-3)-glucan and WIG may be attributed to their molecular size as sulfated α-(1-3)-glucans have much lower size (ranging from 1.2 to 9.0 kDa) while the WIG showed molecular weight beyond 100 kDa (Surenjav et al., 2006). The standard drug (diclofinac sodium) showed complete inhibition of denaturation at 400 μg/ml concentration. Calculated IC_{50} values of anti-inflammatory activity for sulfated α-(1-3)-glucan is 111.55 μg/ml, while for standard drug, diclofinac sodium, was noticed to be 61.70 μg/ml. Similarly, Smiderle et al. (2008) studied anti-inflammatory activity of $(1\rightarrow3)$, $(1\rightarrow6)$-linked β-glucan isolated from *Pleurotus pulmonarius* in rodent model and reported ID_{50} value of 1.19 mg/kg. In view of the reported nutraceutical and probiotic benefits of glucans and the observed anti-inflammatory activity, the glucans will be effective as anti-inflammatory compounds compared to commercially available drugs.

3.8. *Fibrinolytic activity*

The sulfated α-(1-3)-glucans and WIG imperative role of fibrinolytic activity was investigated by using the fibrin clot. Fig. 5,

Table 3
Effect of glucans and sulfated glucans on heat induced protein denaturation.

Concentration (μg/ml)	% Inhibition of denaturation of albumin protein		
	Diclofinac sodium	WIG[a]	Sulfated glucans
100	81.03448 ± 0.312	36.2069 ± 0.387	44.82759 ± 0.215
200	89.65517 ± 0.240	39.65517 ± 0.309	65.51724 ± 0.241
300	92.24138 ± 0.179	39.65517 ± 0.289	68.96552 ± 0.192
400	100 ± 0.017	39.65517 ± 0.293	75.86207 ± 0.297
500	100 ± 0.000	39.65517 ± 0.316	82.75862 ± 0.302

[a] Water insoluble glucans.

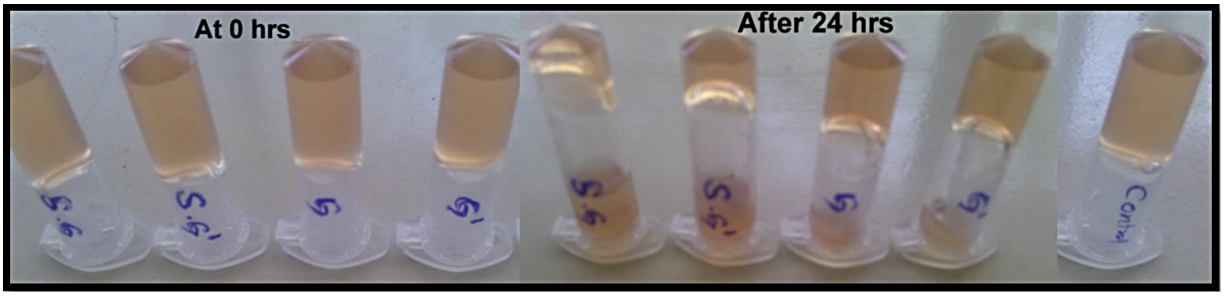

Fig. 5. Fibrinolytic activity of water insoluble glucans and sulfated glucans.

clearly demonstrates that sulfated α-(1-3)-glucans were potential fibrinolytic agents than WIG. Initial observation revealed that both WIG and sulfated α-(1-3)-glucans did not show any fibrinolytic activity up to 6 h incubation. However, a progressive lytic property of fibrin clot was noticed with sulfated α-(1-3)-glucans and complete lysis of fibrin clot was observed within 24 h, where as partial lysis of fibrin clot was observed with WIG even after 24 h incubation (Fig. 5). Such observed difference in lysis of fibrin clot may be attributed to the interactive behaviour of sulfated α-(1-3)-glucans and WIG due to the ionic surface mediated diffusability difference along with variation in molecular weight. This is because, WIG are characterized with higher molecular weight (Surenjav et al., 2006) while sulfated α-(1-3)-glucans posses the molecular weight ranging from 1.2 to 9.4 kDa (Fig. 3). Alban and Franz (2001) also reported similar observations on the fibrinolytic potency of the sulfated α-(1-3)-glucans where it was noticed that molecular weight (MW), the degree of sulfation (DS), the sulfation pattern and the polysaccharide basic structure are decisive parameters for fibrinolytic potency. In fact, the above conclusion may be supported by the commercially available fibrinolytic agents such as urokinase (UK), streptokinase (SK) where molecular weight mediated difference in fibrinolytic activity was reported (Dubey, Kumar, Agrawala, Char, & Pusp, 2011). In addition, sulfated α-(1-3)-glucans mediated fibrinolytic activity is more advantageous to that of commercially available fibrinolytic agents as these glucans are probiotic in nature showing the health benefits such as nutraceutical, immunomodulating, etc. (Jong & Birmingham, 1992). Further, UK and SK mediated fibrinolytic activity requires plasmin proteins as tissue plasminogen activator which are essential for fibrinolysis (Deng et al., 2010). To evaluate whether sulfated α-(1-3)-glucans mediate fibrinolysis require the plasma proteins as activator, a few experiments were performed with and without plasma proteins in the reaction mixture. The results indicated that both clots with and without plasma proteins showed lysis of fibrin clot (data not shown) suggesting that the sulfated α-(1-3)-glucans can also act as tissue plasminogen activators indicating an additional benefit similar to that of serralysin proteins reported by Lakshmi and Prakasham (2013). This is the first report on fibrinolytic activity by sulfated α-(1-3)-glucans.

4. Conclusions

S. mutans cell wall associated WIG were isolated and converted to water soluble form by sulfation. Both these glucans (WIG and sulfated α-(1-3)-glucans) were characterized for their characteristic bonding (α-(1-3)-) by FT-IR spectroscopy. These glucans showed good water solubility and revealed molecular weight ranging from 1.2 to 9.0 kDa up on sulfation. Sulfated α-(1-3)-glucans exhibited potential bioactivity especially anti-bacterial, *in-vitro* anti-inflammatory and fibrinolytic properties. The observed effective antibacterial property of sulfated α-(1-3)-glucans against standard, streptomycin, with respect to gram negative pathogenic bacterial strains suggests sulfated α-(1-3)-glucans may be used as alternative medicine for commercially available antibiotics in treatment of gram negative pathogenic bacterial diseases. The solubilization of fibrin clot with sulfated α-(1-3)-glucans is characterized without the involvement of tissue plasminogen activator contrary to commercially available fibrinolytic agents such as urokinase and streptokinase suggesting the potential benefits of these in health sector.

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