Edifying the strategy for the finest extraction of succinoglycan from Rhizobium radiobacter strain CAS

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Abstract Succinoglycan is an industrially important exopolysaccharide (EPS) that is produced by certain bacteria. There are several procedures to extract this EPS, though the efficiency of all the available procedures is questionable and any improvement in the extraction efficiency can greatly benefit the industry. Here we emphasize on optimization and development of new modus operandi to efficiently extract succinoglycan from liquid bacterial culture. Also, we studied the effect of different extraction methods on production, rheological and structural properties of succinoglycan. Eighteen different chemical and physical methods were tested for succinoglycan extraction from Rhizobium radiobacter CAS isolates with the principle of extracting EPS by precipitating it, where only eleven methods could precipitate the succinoglycan. Comparing the extraction yield of all methods, biopolymer extracted by acetone (3014 mg/L) was maximum followed by cetyl-trimethyl-ammonium-bromide (CTAB 2939 mg/L) and vacuum evaporation (2804 mg/L) methods. Upon comparison of rheological property of recovered succinoglycan, it was found that at shear rate 50 s⁻¹ EPS recovered using acetone and CTAB methods tends to make the solution highly viscous with a viscosity of 150 and 146 mPa s, respectively. In agreement with these results, power law equation showed that EPS extracted by acetone and CTAB had high consistency index (k) and low flow behavior index (n). The current results showed that the physicochemical methods for EPS extraction significantly affect the structural composition of, though succinoglycan extracted using acetone and CTAB showed minimum structural abrasion.

Keywords Exopolysaccharide · Extraction · Power law · Rhizobium · Succinoglycan

Introduction

Exopolysaccharide (EPS) is high molecular weight compounds formed by polymerization of homo- and heteromonomeric sugar residues with diverse commercial applications in industries owing to their virtues and physical properties such as pseudoplasticity, thixotropy, viscosity, gelling and emulsifying activity [1, 2]. EPS is referred source of “green” chemistry and synthesis for its valuable properties in view of potential applications in food, cosmetics and pharmaceutical industries [3, 4]. To list few, alginate, chitosan, curdlan, dextran, levan, pullulan, succinoglycan xanthan, etc. are some commercially important microbially derived EPSs.

Succinoglycan is an economically important high molecular weight EPS produced extracellularly by Sinorhizobium, Rhizobium, Agrobacterium, Alcaligenes and Pseudomonas [5–13]. It is commercially used in cosmetics and home care products, fertilizer formulations, pharmaceuticals, food industries, for enhanced oil recovery as an emulsifying agent, gelling agent, stabilizing agent,
texting agent and thickening agent. All such properties of succinoglycan are attributed due to its high consistency under extreme operational condition such as high temperature, salt or ionic concentration, pressure and high shear rate [9, 14–18].

Considering the commercial importance of microbial EPS, numerous universal extraction methods are available for the precipitation, separation and extraction of microbial EPS [19]; however, under different scenario including culture conditions and type of EPS, the extraction procedure has to be modified. Key points considered in EPS extraction include (1) approaches to release and precipitate EPS, with minimal structural abrasions, and (2) minimal alteration in characteristics and property of extracted EPS [20–23]. Owing to this, different extraction procedures have to be employed to extract different EPS. For instance, optimized extraction protocol for xanthan may not work efficiently for succinoglycan. Regardless of several EPS extraction techniques in the literature, it is necessary to optimize modus operandi from the standpoint of yield and composition. Also, after optimizing the extraction procedure for a particular EPS, the quality and structural abrasions of extracted EPS have to be accessed [24, 25].

Under present study, we conducted an extensive optimization study of extraction procedures for succinoglycan extraction from culture suspension of Rhizobium radiobacter strain CAS (GenBank accession number: KJ191122.1). The succinoglycan produced by R. radiobacter strain CAS was earlier characterized by FT-IR and NMR spectroscopy and was found to be composed of glucose and galactose in a molar ratio of 6.99:1 as confirmed by HPAEC and published by Andhare et al. [26]. Wide range of chemical solvents, salts and physical methods were used individually or/and in combination to precipitate and extract out the succinoglycan from the culture broth [6]. The overview of each extraction scheme is illustrated in Fig. 1.

Methodology for extraction of succinoglycan using chemical methods includes separation of cell biomass by centrifugation at 9400 × g at 25 °C for 10 min, followed by the addition of two volumes of chemical solvents in the culture supernatant to extract succinoglycan by precipitation, which was recovered using centrifugation at 9400 × g for 5 min at room temperature. Thirteen chemical methods to extract succinoglycan from culture supernatant included addition of two volumes chilled acetone [27], two volumes chilled ethanol [8], two volumes chilled methanol [28], two volumes chilled iso-propyl alcohol [11], two volumes chilled n-propyl alcohol, cetyl-trimethyl-ammonium-bromide (CTAB 2% w/v, 100 ml) [29], two volumes chilled butanol, two volumes chilled iso-amyl alcohol [12], sodium hydroxide (NaOH 1 M, 100 mL) [30], glutaraldehyde (Sigma 25%, 100 mL) [31], formaldehyde (36.5%, 100 mL) [32], ethylenediaminetetraacetic acid (EDTA 2%, 100 mL) [33] and formaldehyde–NaOH (50 mL each) [30]. A diagrammatic summary of all the aforementioned eleven extraction methods is shown in Fig. 1. The chemical method which showed maximum precipitation of succinoglycan was further optimized by combining physical treatments to the original chemical extraction procedures.

Selection of the best succinoglycan extraction procedure combining physical treatments to chemical method

Four-day-old culture of R. radiobacter CAS growing in liquid Bushnell Hass broth containing sucrose as a carbon source was used. Briefly, cell biomass was removed from the culture broth by centrifugation at 9400×g at 25 °C for 10 min to get cell-free culture supernatant. Thereafter, five physical treatments were individually given to culture supernatant, which included (1) boiling [31], (2) heating [21], (3) sonication, (4) sonication–boiling and (5) vacuum evaporation [34]. After providing physical treatment to the culture supernatant, succinoglycan was extracted by precipitation, using twice the volume of acetone. Here we opted to use acetone because it yielded maximum extraction of succinoglycan from all the previously accessed chemical solvents.

The exopolysaccharide precipitated was collected by centrifugation at 9400×g for 5 min at room temperature...
and dried in hot air oven for overnight at 50 °C [35]. Dried EPS was grind to fine powder and preserved for rheology and structural elucidation studies.

**Rheology studies of extracted succinoglycan**

Different extraction conditions by physical and chemical methods may alter the rheological behavior of extracted succinoglycan.
succinoglycan. As a consequence, the molecular conformations and rheological profile are influenced by the harsh treatments employed to extract EPS [13]. Rheological analysis will provide an insight to the abrasion caused to succinoglycan by the harsh treatments. The ideal extraction procedure is one which causes minimum abrasion to the complex structure of EPS. Accordingly, rheological analysis of extracted succinoglycan was carried out in Anton Paar Rheolab QC using cup and bob geometry. Viscosity of all the succinoglycan samples extracted using different methods was measured.

Aqueous solution of 1% (w/v) EPS in distilled water was prepared and was left overnight in refrigerator for complete hydration. This aqueous solution of EPS was used as a sample for rheological analysis. A shear rate sweep from 0.01 to 1000 s$^{-1}$ [18] was applied for 5 min with 60 points at a time interval of 5 s each, and rheological data were collected at 25 °C. The RheoPlus software from Anton Paar was used for operating the instrument and recording results. The relationship between apparent viscosity and shear rate was studied by computing the consistency coefficient ($k$) and flow behavior index ($n$) and overall yield of EPS is inevitable from the industrial perspective. Under present study, an effort is made to enhance the overall yield of succinoglycan from a microbial source. After optimizing the yield of succinoglycan, the effect of extraction procedure on the structural abrasion and rheological behavior was studied. The comprehensive research was carried involving succinoglycan extraction from our strain $R$. radiobacter strain CAS (GenBank accession number: KJ191122.1), using eighteen different methods as shown in Fig. 1. Briefly, succinoglycan was precipitated from the 4-day-old culture supernatant of $R$. radiobacter strain CAS grown in Bushnell Hass broth containing sucrose as carbon source (10 gm/L) using twice the supernatant volume in all studies for equal comparison. We have previously proved that $R$. radiobacter strain CAS produced EPS.

We found that yield of succinoglycan was poor when butanol, iso-amyl alcohol, NaOH, glutaraldehyde, formaldehyde and formaldehyde–NaOH were used. The yield of succinoglycan extracted from $R$. radiobacter strain CAS by eleven processes is summarized in Fig. 2. Outcomes showed that the EPS production was importantly dependent upon the extraction method. Higher quantity of EPS was extracted when acetone (3014 mg/L) followed by CTAB (2939 mg/L) and vacuum evaporation (2804 mg/L) was used as precipitating agents. Total quantity of EPS produced by acetone extraction was 5.8 times higher than the most commonly used precipitating agent, ethanol (519 mg/L). The difference in succinoglycan yield in eleven physicochemical methods varied in the range of 519–3014 mg/L. Ethanol is widely reported that ethanol can efficiently precipitate succinoglycan with the volume required being thrice or more to the volume of supernatant, whereas here we showed that acetone was required in much lesser quantity and yield was several folds high in comparison with ethanol.

Firstly, we suggested that acetone extraction could prove efficient for EPS produced by $Rhizobium$ sp. Secondly, when succinoglycan was extracted by adding one tenth of the culture supernatant volume of CTAB (2% w/v), the yield was high. To the best of our knowledge, this is the first report suggesting the use of CTAB to extract biopolymer from $R$. radiobacter. Lastly, vacuum evaporation also presented potential prospective for EPS extraction, as it reduced the overall acetone volume required to precipitate EPS. It extracted 2804 mg/L EPS which was higher than rest of the methods employed in the present study. Thus, we conclude that acetone precipitation aided with vacuum evaporation is the best extraction procedure to recover succinoglycan. Furthermore, this procedure also stands out to be economical as large amount of acetone is not required to achieve efficient extraction.

### Results

#### Extraction of succinoglycan by physicochemical methods

Yield of EPS from the microbial source is important for industry. So any progress which can enhance the recovery...
Rheology studies of extracted EPS

The flow curves of succinoglycan for different extraction methods reported the effect of shear rate on apparent viscosity in Fig. 3. With increase in shear rate, the apparent viscosity considerably decreased indicating shear thinning (pseudoplastic) behavior. Among the eleven different methods tested, precipitation with acetone and CTAB was highly efficient in extraction of succinoglycan and the extracted succinoglycan using these methods had greater viscosity. This indicates that precipitation with acetone and CTAB does not erode the complexity of succinoglycan. At shear rate 51, viscosity produced by EPS extracted by acetone and CTAB methods was 150 and 146 mPa s, respectively. The coefficients of this equation along with regression coefficient ($R^2$) for extracted all succinoglycan samples are presented in Table 1. The relevance of power law model and high level of relation between measuring points to evaluate flow properties of extracted EPS were suggested by regression coefficient ($R^2$) with higher range of 0.95–0.99. The consistency coefficient ($k$) defines the inclusive range of viscosities of resulted EPS from different procedures. The highest consistency index ($k$) and the lowest flow behavior index values ($\eta$) were reported in succinoglycan extracted by acetone and CTAB. Our results showed the effect of extraction methods influenced consistency index and elevated the biogum precipitation where succinoglycan extracted using acetone and CTAB showed $k$ value equal to 2760.9 and 2871.9, respectively. Flow behavior indices, on the other hand, had decreased significantly for acetone (0.241) and CTAB (0.235) methods. The $\eta$ values for all extracted succinoglycan solutions were between 0 and 1, ranging from 0.235 to 0.743, revealing shear thinning behavior as lower values of exponent $\eta$ (close to zero) are more pseudoplastic products. The flow behavior coefficient denotes the degree of pseudoplastic nature of extracted EPS by different approaches. To our knowledge, this is the first study explaining the effect of

Fig. 2 Extraction of succinoglycan using different physicochemical methods. Extraction by acetone, vacuum evaporation and CTAB resulted in high EPS yield than other methods. [Analysis of variance (ANOVA) was carried out to identify significant extraction enhancement as compared to the minimum yielding method (MYM) (i.e., ethanol extraction) the EPS extraction values were compared at significance levels of 5, 1 and 0.1% LSD]. $p$ value has been calculated using one way ANOVA and its interpretation is as follows: ns ($p$ value greater than 0.05), non-significance as compared to the minimum yielding method (MYM) (i.e. ethanol extraction); *($p$ value between 0.05 and 0.01), significant at 5% as compared to MYM; **($p$ value between 0.01 and 0.001), significant at 1% as compared to MYM; ***($p$ value less than 0.001) significant at 0.1% as compared to MYM.

Fig. 3 Effect of extraction method on rheology of CAS EPS (CTAB cetyl-trimethyl-ammonium-bromide)
physicochemical extraction methods on consistency index ($k$) and flow behavior index values ($\eta$) of succinoglycan.

### Structural characterization of extracted EPS by FT-IR analysis

Succinoglycan extracted using acetone, CTAB and vacuum evaporation showed efficient biogum production, so only these samples were analyzed using FT-IR. Also, FT-IR spectra of these samples were compared with the spectra of standard succinoglycan. Comparison of FT-IR spectra of extracted succinoglycan with standard succinoglycan is illustrated in Fig. 4 to analyze extraction methods capability to uphold the EPS structure and its properties. FT-IR spectra of acetone-extracted succinoglycan, CTAB-extracted succinoglycan and standard succinoglycan are superimposable as these spectra are similar. These findings suggest that acetone and CTAB extraction methods did not affect the glycosidic bond and sugar ring of carbohydrate; however, succinoglycan extracted using vacuum evaporation method had dissimilar spectra as observed by its distorted spectra with weak peaks. Except for vacuum evaporated succinoglycan, other spectrums had pronounced band between 3399 and 3405 cm$^{-1}$ characteristics for O–H symmetrical stretching vibration for carbohydrate compounds and involved in EPS water solubility according to data explained by Karbowiak et al. [38]. The detailed band assignment of main absorption peaks is shown in Table 2. Comparison of spectra for acetone and CTAB with succinoglycan showed that extraction methods had no effect on functional groups and structure of extracted biopolymer and both spectra comprised hydroxyl and carboxyl which correspond mostly to occurrence of carbohydrate as shown in Fig. 4 and Table 1. On the contrary, FT-IR of EPS extracted by vacuum evaporation had weak signals and prominent carbohydrate peaks were absent indicating the structural abrasion. Consistent with this, rheological analysis of vacuum evaporation precipitate EPS had high flow behavior index ($\eta$) and low consistency index ($k$) confirming the effect of extraction method on conformation and property of exopolymers.

### Discussion

EPS is a commercially important product as it is widely used in food and pharmaceutical industry though they are majorly used as gums, stabilizers, thickeners, emulsifiers, etc. EPS is a polymer of monosaccharides produced by microbes by utilizing available sugars from the environment [39]. Bacteria accumulate this EPS forming a layer called as capsule above cell wall. Bacteria produce diverse EPS with different complexity and structure. Property of EPS to act as a gelling agent, thickeners, etc. depends on its

![Fig. 4](image-url)
structure and molecular weight [4]. Thus, while extracting EPS from a microbial source, distortion in its structure should be avoided. The technique which can extract EPS without causing any significant harm to its structure is commercially viable [40]. Also, at a commercial platform the technique should provide high yield along with maintaining structural integrity of EPS [41]. Under present study, once such effort is made to enhance the extraction yield of succinoglycan without causing any significant distortion in its structure.

It is well known that strains belonging to *Rhizobium* produce succinoglycan [36, 42, 43]. Strains of *Rhizobium* possess 11 essential genes (exoY, exoA, exoL, exoM, exoO, exoU, exoW, exoP, exoQ and exoT) which are needed to produce succinoglycan [44]. Under present study, we used one such strain *R. radiobacter* strain CAS which produced succinoglycan from which we performed extraction of succinoglycan using several chemical and physical techniques.

Out of 11 extraction procedures employed, acetone, CTAB and vacuum evaporation yielded efficient extraction of succinoglycan. Even though use of ethanol is well cited and most routinely used precipitating agent for extraction of microbial EPS [40, 45], we showed that it is not an efficient extracting agent specifically for succinoglycan. It is widely reported that ethanol can efficiently precipitate several microbial EPS with the volume required being thrice or more to the volume of supernatant [41], whereas here we showed that acetone was required in much lesser quantity and yield was several folds high as compared to ethanol. Use of CTAB to extract microbial EPS in general is well known [29], though here we showed the exact proportion of CTAB required for optimum extraction specific for succinoglycan. We also showed that out of several EPS extracting agents, CTAB is one among the best for microbial succinoglycan. The third best technique in terms of succinoglycan extraction was vacuum evaporation. This is the first report for the use of vacuum evaporation to specifically extract succinoglycan from microbial source. Similar use of vacuum evaporation to recover EPS for microbial succinoglycan was performed by Polak-Berecka et al. [46] though the authors did not compare the yield of the extracted EPS with other methods, nor the extracted EPS was succinoglycan.

Only studying the yield of succinoglycan using different procedures is not sufficient so we studied the physical property of viscosity and rheology exhibited by succinoglycan extracted using the most efficient extraction procedures (extraction using acetone, CTAB and vacuum evaporation). The reason for performing such experiment is to understand the quality (in terms of polysaccharide complexity) of extracted succinoglycan, as the treatments used to extract succinoglycan might cause physical harm to the polymeric structure of EPS which is directly reflected on the complexity of the structure. More the physical harm to the EPS, lesser would be the structural complexity and lesser would be the viscosity and vice versa. So, greater the viscosity exhibited by extracted EPS, the lesser is the structural abrasion caused by the extraction treatments [47]. It is important in industrial point of view to know which extraction method yields these properties as rheological assets affect the thickening, gelling and flow behavior and processing operation of products [47].

### Table 2 Structural characterization of extracted EPS by acetone and CTAB methods and comparison with standard succinoglycan by FT-IR analysis

| Wavenumber (cm⁻¹) | Band assignment                                                                 | References                  |
|------------------|---------------------------------------------------------------------------------|-----------------------------|
| 3408             | Stretching vibration of –OH group of sugar polyhydroxyl groups                  | Yan et al. [52]              |
| 2921             | –CH stretching of CH₂/CH₃ groups in carbohydrate backbone                        | Liu et al. [53]              |
| 1726             | C=O stretching of acetyl ester                                                  | Castellane et al. [48, 49]   |
| 1402             | –COO⁻ asymmetrical stretching vibration of carboxylic groups                    | Ruiz et al. [54]             |
| 1066             | C–C or C–O stretching vibration or asymmetrical C–O–C stretching band resulting from sugar backbone | Botelho et al. [56]          |

Key functional groups observed from FT-IR spectra of extracted EPS were studied.
agreement with previous published studies by Castellane et al. [48, 49]. The flow behavior coefficient denotes the degree of pseudoplastic nature of extracted EPS by different approaches, conferring distinctive viscosity properties to manipulate and control the rheological nature of aqueous solutions as related by Moretto et al. [50]. The succinoglycan extracted using acetone and CTAB showed enhanced k value and the lowest n value. According to the finding of Gómez-Díaz and Navaza [51], enhanced k value is associated with high water binding ability, and this means succinoglycan extracted using acetone and CTAB could efficiently bind water and could remain hydrated. This property is attributed only if the extracted EPS is complex and unharmed by the extraction procedures as small and simple microbial EPS cannot bind water and exhibit high viscosity [50]. However, vacuum evaporation to precipitate EPS had high flow behavior index (n) and low consistency index (k) which shows structural abrasion. To our knowledge, this is the first study explaining the effect of physicochemical extraction methods on consistency index (k) and flow behavior index values (n) of succinoglycan.

Lastly, succinoglycan extracted from acetone, CTAB and vacuum evaporation methods was analyzed by FT-IR spectroscopy to study the structural abrasion. On comparing the spectra of these extracted succinoglycan samples with standard succinoglycan, the signature peaks were unaltered in the succinoglycan extracted using acetone and CTAB [36]. However, this was not true in case of succinoglycan extracted using vacuum evaporation. Thus, acetone and CTAB can efficiently extract succinoglycan without causing any significant structural abrasion.

This study reported that extraction methods significantly affect the amount and chemical composition of succinoglycan extracted from R. radiobacter strain CAS. Methodology consisting acetone and interestingly CTAB extraction was the most efficient of all other methods evaluated in this study extracting 3024 and 2939 mg/L of succinoglycan, respectively. Cationic nature of CTAB found effective in extraction of an anionic CAS succinoglycan. Rheological studies to observe further decipher that acetone and CTAB used for extraction were the best methods for succinoglycan precipitation produced by Rhizobium sp. in consideration with industrial implication as rheological properties are significantly valuable in application point of view. The power law model well elucidated that succinoglycan extracted by acetone and CTAB methods depicted increased viscosity behavior as non-Newtonian shear thinning fluid complemented by small flow behavior index (n) and high consistency value (k). FT-IR observation also suggests that acetone and CTAB were the best methods for EPS extraction produced by Rhizobium sp. as they did not cause any structural damage to succinoglycan.

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**Compliance with ethical standards**

**Conflict of interest** All the authors of the manuscript declare that they have no conflict of interest.

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