Levan Production in Shake Flask and Fermenter - Influence of Feeding Strategy on Levan Yield and Molecular Weight Distribution

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Abstract. Effect of feeding strategy on levan production was studied in a shake flask and a 5 L lab scale fermenter. In a shake flask, levan specific substrate yield ($Y_{P/S}$) increased from 0.35 g levan/g sucrose to 0.48 g levan/g sucrose with repeated batch feeding. In a 3 L reactor, levan productivity of 7.18 g/L h was obtained with the fed-batch mode of fermentation. The gel permeation chromatography results indicate that higher initial sucrose concentration under the fed-batch mode operation resulted in the formation of low molecular weight fractions (4 and 10 kDa). Thus, fed-batch fermentation favors levan production resulting in higher yield and productivity and also affects the molecular weight distribution of the biopolymer.

Keyword. Circular economy, Feeding strategy, Levan, Levan type fructooligosaccharide, Molecular weight distribution

1 Introduction

Levan is a fructose based biopolymer produced by both microorganisms and plants. Levan is produced by a variety of microorganisms from sucrose via the action of the enzyme levansucrase. It consists of two fructan molecules linked by β-(2, 6) and β-(2, 1) linkages within the core and branch chains, respectively, and carry D-glucosyl residues at its terminal end. Levan has been widely produced by the microorganisms belonging to genera viz., *Pseudomonas, Bacillus, Halomonas, Erwinia, Paenibacillus, Streptococcus, Zymomonas, and Acetobacter* [1]. The physicochemical properties, degree of polymerization, and molecular weight of levan strongly influence biological activities as well as biotechnological applications. It is considered that the size and molecular weight of levan differ on the microbial source and substrate concentration maintained during fermentation [2].

Levan is solely produced using sucrose as substrate, which is obtained from agricultural activities. Thus, it is considered as a value addition to agricultural processing industry to boost its economy. In our recent study, we have demonstrated that levan could be directly obtained from sugarcane juice without need for refining it. Thus, the process is suitable for the circular economy model [3]. Levan, oligolevan, and levan-type fructooligosaccharides (LFOS) find their application in the consumer sector ranging from food industries, cosmetic industries, pharmaceutical industries, etc. It has also gained increased research focus over the last few years due to its anti-tumor, immuno-modulating, and prebiotic activities. Low viscosity, strong adhesivity, high water, and chemical holding capacity, self-assembling ability, high water-solubility, non-toxicity, and biodegradability make the polymer very attractive in the food and biomedical sectors [4]. About 60% of the theoretical yield (0.31 g levan/g sucrose) has been obtained so far using *Bacillus subtilis* [5]. In comparison, Shih *et al.*, (2005)[6] reported a yield of 0.247 g levan/g sucrose using *Bacillus subtilis* (Natto) at a concentration of 200 g sucrose/L. When the initial concentration of sucrose during batch fermentation was increased further to 250 g sucrose/L, the yield of levan obtained using different strains of *Bacillus subtilis* (*Bacillus subtilis* var. natto, *Bacillus lentus* V8 strain, and *Bacillus subtilis* (Natto) Takahashi) reported were 0.166, 0.224 and 0.230 g levan/g sucrose, respectively [7-9]. These results suggest that increased substrate concentration, results in low yield and productivity which could probably be due to substrate inhibition. To overcome the issue of inhibition at high substrate concentration, fermentation using fed-batch mode of operation could be a promising alternative to traditional batch fermentation to increase the levan yield and productivity.

Fed-batch fermentation is one of the most popular methods used to attain high cell density, product concentration, and production rate [10]. This mode of operation helps to overcome substrate inhibition and catabolite repression. During fed-batch fermentation, the

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concentrations of the substrate in the medium are varied by adjusting the frequency of substrate feeding intervals. The main success of the fed-batch process is the designing of a feeding strategy that satisfies the demands of cells to production of the desired product [11]. The most efficient feeding strategy is determined by considering specific product formation rates as a criterion to eventually achieve increased production levels. On comparing the production process with the conventional batch fermentation process, the fed-batch fermentation along with substrate inhibition significantly promotes the productivity of various products such as lactic acid, formic acid, lipid and PHB [12-15]. A repeated batch fermentation strategy is a constructive production route that has several advantages, such as short separation time, higher substrate consumption, and increased biomass and product productivity. The effect of levan molecular weight distribution at different modes of reactor operation- batch is not reported to date to the best of our knowledge. Also, data on feeding strategy on levan yield and molecular weight distribution are sparingly available in the literature. Hence, the current study aims to evaluate the effect of feeding strategy on levan specific yield and its molecular weight distribution.

2 Materials and Methods

2.1 Reagents and microorganisms for culture medium

The strain *Bacillus subtilis* MTCC441 was purchased from MTCC-Microbial Type Culture Collection, Chandigarh, India was used in this study. Isopropanol used for levan recovery was obtained from SRL chemicals, India. All other reagents including sucrose used in this study were of analytical grade (HiMedia, India). The seed culture was prepared using Luria Bertani (LB) broth and incubated at 37 ºC overnight in an orbital shaker at 150 RPM. The glycerol stock of this strain of *Bacillus subtilis* was maintained at -80 ºC.

2.2 Repeated fed-batch in shake flask

Batch production of levan was carried out in 250 mL Erlenmeyer's flask using 10% (v/v) of overnight culture as inoculum. The composition of production media was as follows (in g/L): sucrose - 100, yeast extract - 2, ammonium sulphate ($(NH_4)_2SO_4$) - 3, potassium dihydrogen phosphate (KH_2PO_4) - 1, manganese sulphate ($MnSO_4$) - 0.2, and magnesium sulphate ($MgSO_4.7H_2O$) - 0.6. The initial pH of the medium was set to 7.0 by the addition of 1.0 N HCl or 1.0 N NaOH before the start of the sterilization process. Batch fermentation is carried out under previously optimized conditions 37 ºC, 150 RPM, and 20 h [16, 17].

Repeated fed-batch studies were conducted out in 500 mL Erlenmeyer's flask with a 100 mL working volume. The same fermentation conditions as mentioned above were used in the fed-batch mode also. The process was started with 100 mL production medium with an initial sucrose content of 100 g/L. Subsequently, at the late exponential phase (after 24 h), 50 mL culture broth was withdrawn and the flask was topped up with an equal amount of fresh substrate containing 200 g/L sucrose. The remaining cells in the flask serve as inoculum for the next batch. The pH of the culture was set to 7.0 using 1N NaOH. This procedure was repeated every 24 h for 5 days. Throughout the study, the culture was maintained at 37 ºC, and 150 RPM. The concentrations of residual sugar, levan, and biomass in the harvested culture were estimated. Experiments were conducted in triplicates and the means were compared using Tukey's test at $\alpha = 0.05$.

2.3 Fermenter studies

After completing shake flask studies, fermentation studies were performed in a 5 L bench-scale fermenter with a working volume of 3 L (Make : M/s Lark Biotech, India). The fermenter was fitted with two six-bladed Rushton impellers. The agitation speed, aeration rate, and temperature were maintained at 250 rpm, 1 LPM, and 37 ºC, respectively. The fermentation pH was maintained at 6.0 by the addition of 2M NaOH/2N HCl solution as required. Fermentation was carried out for three different sucrose feeding modes viz:
(1) batch mode - initial sucrose concentration 100 g/L
(2) batch mode - initial sucrose concentration 250 g/L and
(3) fed batch mode - here initial sucrose content of 100 g/L was taken and after 12 h a pulse addition was made to raise the concentration to 250 g/L. That is after 12 h (late exponential phase), when the residual sugar concentration drop below ~10 g sucrose/L, 1 L of culture broth was removed from the reactor and an equal volume of fresh feed containing approximately 75% sucrose concentration was added to increase the residual sugar concentration in the reactor to 250 g sucrose/L.

Considering the scale of operation, for 5 L reactor studies an inoculum size of 5% was used. Fermentation was carried out at 37 ºC and 250 RPM. The samples were taken after, every 4 h, until 48 h to estimate biomass concentration, levan production, and residual sugar concentration.

2.4 Biomass recovery and levan isolation

For biomass recovery, the culture samples were centrifuged at 8000 RPM for 10 min and the pellets were collected. The pellet was dried overnight at 60 ºC for biomass determination. Levan was precipitated from the cell-free supernatant following a pH adjustment to 9.0. After adjusting pH, the supernatant was added to ice-cold isopropanol in a 1:3 ratio to precipitate the levan biopolymer. The precipitate was then centrifuged at 8000 RPM for 10 minutes and the obtained pellet was dried at 60 ºC to obtain crude levan. Purification of levan was carried out by re-precipitation using IPA (1:3) followed by centrifugation. Reprecipitation was repeated twice to ensure maximum removal of impurities. The pellet was then re-suspended in hot water and purified using dialysis. The water was changed two times a day for 3 days. This purified levan was freeze-dried and stored at 4 ºC for future use [16, 17].

2.5 Determination of intrinsic viscosity

The intrinsic viscosity [η] of the levan solution (0.25-10 g/dL) was determined in triplicates using a Brookfield Viscometer (LV DV 2+ pro deployed with extra S18 spindle). 10 mL of the levan solutions were placed to a sample holder in a viscometer and at different shear rates, apparent viscosity was measured at room temperature. The apparent viscosity (η) of the sample was changed to relative viscosity (η_{rel}) and specific viscosity (η_{sp}) using eq. (1) and eq. (2).

$$\eta_{rel} = \frac{\eta}{\eta_s} \quad (1)$$

$$\eta_{sp} = \eta_{rel} - 1 \quad (2)$$

where η_s is the solvent viscosity.

The intrinsic viscosity of the diluted levan solutions was calculated using Huggins model (eq. 3) and Kraemer model (eq. 4) [18] where solutions were prepared to give relative viscosity ranging from 1.2 to 2, and the corresponding specific viscosity range from 0.2 to1.0.

$$\frac{\eta_{sp}}{C} = [\eta] + K_H[\eta]^2 C \quad (3)$$

$$\frac{\ln \eta_{rel}}{C} = [\eta] + K_K[\eta]^2 C \quad (4)$$

Where K_H is Huggins constant, K_K is Kraemer constant, and C is solute concentration.

Intrinsic viscosity can also be determined using Tanglertpaibul - Rao model (eq. 5), Higiro model (eq. 6 and eq. 7) [19, 20].

$$\eta_{rel} = 1 + [\eta]C \quad (5)$$

$$\eta_{rel} = e^{[\eta]C} \quad (6)$$

$$\eta_{rel} = 1/(1 - [\eta]C \quad (7)$$

The conformation of polysaccharides was elucidated from the power law (eq. 8) model parameters [20, 21].

$$\eta_{sp} = aC^b \quad (8)$$

3 RESULTS AND DISCUSSION

3.1 Effect of repeated fed-batch on levan production

The results for levan production using a repeated feeding strategy are shown in Figure 1. We hypothesized that levan production can be improved by increasing the biomass and levansucrase enzyme concentration from the first batch. Upon successive substrate feeding in pulse, the concentration of levansucrase enzyme can be maintained at a relatively higher level and this will enhance the production of levan. The results obtained from this study show that more than 95% of the initial sucrose concentration was consumed within an initial 24 h (Figure 1).

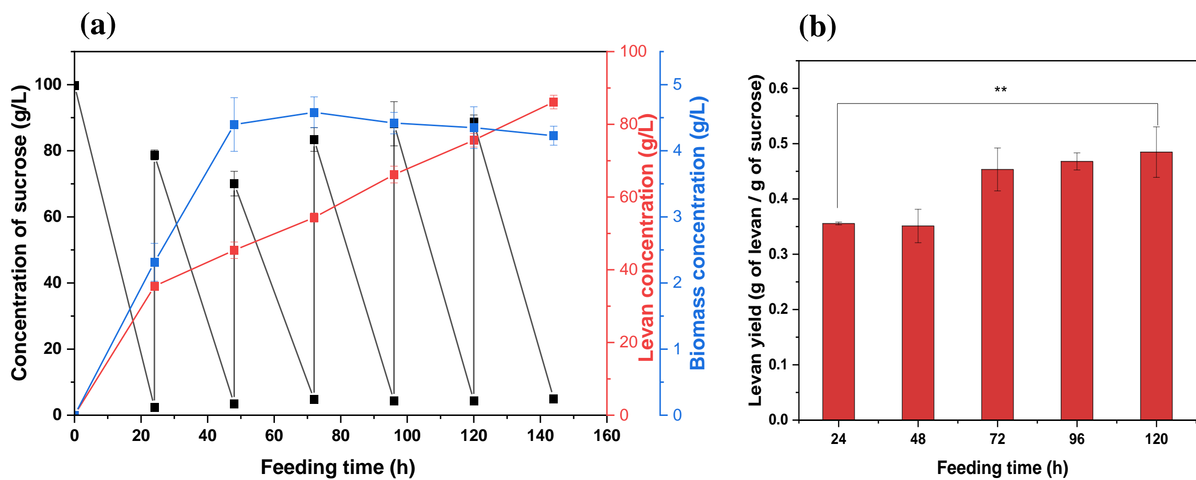

Fig. 1. Effect of cyclic feeding strategy for levan production (a) Time profile for levan concentration, Biomass concentration & Concentration of sucrose (b) Time profile for levan yield on successive feeding of sucrose.

Table 1. Molecular weight distributions of levan from repeated fed-batch fermentation.

Feeding time (h)	F1 (kDa)	F2 (kDa)	F3 (kDa)	F4 (kDa)	F5 (kDa)	F6 (kDa)	F7 (kDa)
24 (Batch 1)	2652.54	ND*	ND	9.16	5.54	3.36	1.56
48 (Batch 2)	2699.57	552.72	ND	8.69	6.25	3.91	1.74
72 (Batch 3)	2891.92	ND	10.65	ND	6.22	3.84	1.80
96 (Batch 4)	2463.45	ND	ND	ND	6.09	3.79	1.81
120 (Batch 5)	2456.25	223.92	ND	ND	6.26	3.83	1.78

* ND- Not determined; F1, F2, F3….F7 = Fraction 1, Fraction 2, Fraction 3…Fraction 7, respectively

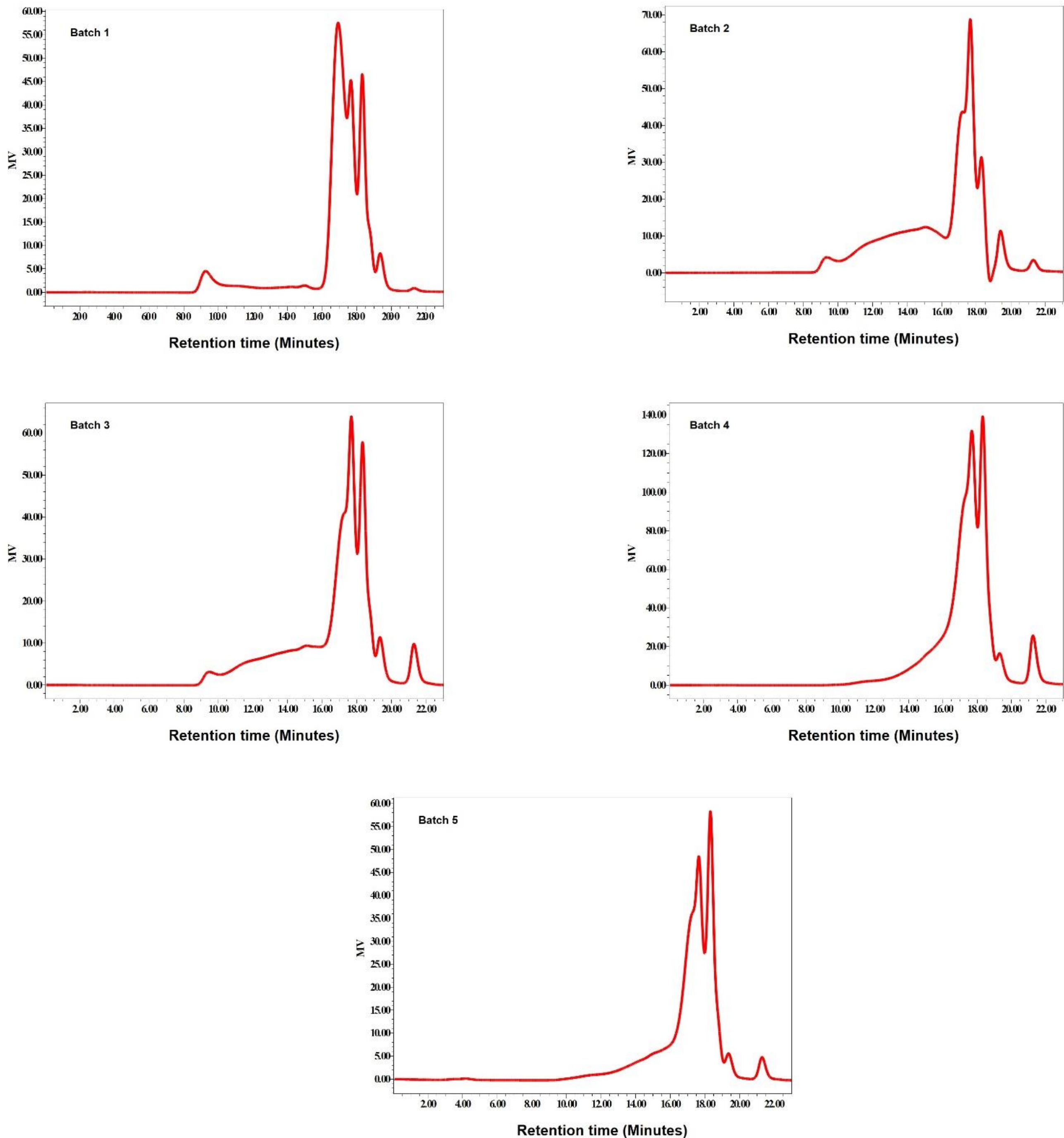

Fig. 2. Gel permeation chromatogram of levan produced from cyclic fed-batch fermentation.

Repeated feeding increased the specific levan yield significantly from 0.35 g/g sucrose (at 24 h) to 0.48 g /g sucrose (at 120 h). This is almost 96% of the maximum theoretical yield (0.50 g levan/g sucrose) possible from sucrose. The levan yields obtained after 96 h and 120 h were statistically the same. Thus, four-step additions could result in maximum specific yield. Similarly, previous reports in the literature on different biopolymers (Poly-Ɛ-lysine, poly-γ-glutamic acid, dextran, EPS) have shown increased yield during repeated feeding of the substrate [22-24] and the results obtained in this study are consistent with previous literature reports.

From the GPC results shown in Figure 2, we can observe that both high molecular (2400 - 2800 kDa) and low molecular (2 - 11 kDa) weight fractions were obtained in repeated fed-batch (Table 1). Results from GPC analysis of levan from batches 1st - 5th indicate that there is no significant change observed in the low and high molecular weight fractions obtained. Whereas levan from the 2nd- 5th batches shows intermediate molecular weight fractions, which could be due to the chain length increase in low molecular weight fractions from the previous batches. The nonprocessive synthesis of low molecular weight levan is mainly due to the oligosaccharide interaction with at least four acceptor binding sites. Due to increasing concentration of levansucrase in the late batches leads to the occurrence of non-processive mechanism resulting in a reduction of molecular weight

[25]. Both levan and LFOS produced can be further fractionated based on their molecular weight as low and high molecular weight levan. Levan biopolymer with a high degree of polymerization (high molecular weight) finds its applications in tissue engineering such as wound dressing. For example, biocompatible levan based hydrogels were formed as drug delivery systems for tissue engineering applications and various medicinal applications [26, 27]. Whereas the low molecular weight fraction of levan i.e., LFOS can be used as a prebiotic in food industries. It has been reported that the intestinal adhesion capacity of probiotic bacteria (*Lactobacillus reuteri* JN101) was improved by levan biopolymer from *Bacillus amyloliquefaciens* JN4 [28]. Also, these types of polysaccharides have gained noticeable importance, such as the ability to enhance the activity of gut microbiota, low caloric sweeteners, non-toxic, and improve the intestinal immune response [29-31].

3.2 Scalability of levan production in bench-scale bioreactor

Results of fermentor studies are shown in Figure 3. Figure 3a shows the sucrose consumption profile at different initial sucrose concentrations. At 100 g/L initial sucrose concentration, about 93.7 % of sucrose consumption was observed within 12 h. In comparison, when the initial sucrose concentration was 250 g/L, similar levels of sucrose consumption (94.8 %) were observed only after 44 h. At an initial sucrose concentration of 100 g/L, the levan yield obtained at the end of fermentation at 12 h is 0.35 g levan/ g sucrose. This indicates that levan results obtained after scaling up from the shake flask to the bench bioreactor showed similar levels of levan yield when operated under batch fermentation. As the initial sucrose concentration was increased to 250 g/L, levan yield increased to 0.46 g levan/g sucrose with less productivity of 2.97 g/ L h at 28 h of fermentation. These results show that substrate inhibition might play a huge role in biomass growth and levan production. To address this issue, a feeding strategy using fed-batch cultivation was devised for reactor studies.

The results obtained from batch fermentation of 100 g/L initial sucrose concentration show, nearly 95 % of sucrose was consumed by *Bacillus subtilis* MTCC441 after 12 h of fermentation. Therefore, at the end of 12 h, about 1.0 L of the fermentation broth was withdrawn and replaced with 1.0 L of 750 g/L (75% w/v) to attain a final sucrose concentration of 250 g/L (25% w/v). Sucrose solution during fed-batch cultivation was added at a feed rate of 30 mL/min. The biomass concentration was increased from 2.67 g/L (12 h) to 5.5 g/L (28 h) Figure 3b.

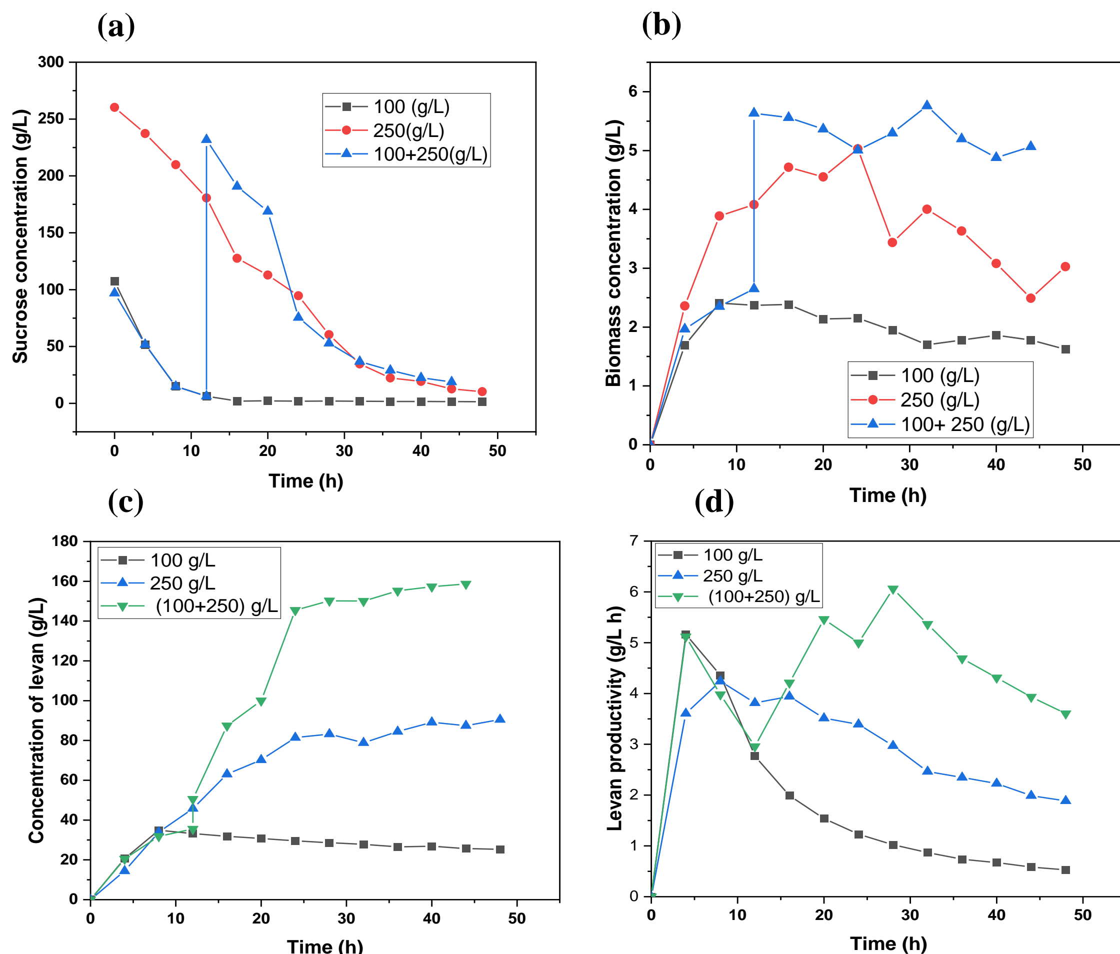

Fig. 3. Effect of feeding strategy on sucrose concentration using batch and fed-batch fermentation. (a) Sucrose consumption rate (b) Biomass concentration (c) Concentration of levan (d) Levan productivity.

After 48 h of the fed-batch mode of fermentation, about 94.8 % of the sucrose consumption was observed for a total sucrose concentration of 350 g/L. Figure 3c shows that levan production follows a similar trend observed in sucrose consumption rate. On comparing batch with fed-batch mode of operation, levan concentration reached 150.03 g/L at 28 h to a total sucrose concentration of 350 g/L with high productivity of 7.18 g/L h. This may be attributed to the presence of biomass and levansucrase enzyme at sufficient levels after 12 h of initial fermentation, which helps in the conversion of sucrose to levan and, in turn, results in higher productivity (Figure 3d). Based on the results obtained from two-stage fermentation, it is evident that the fed-batch fermentation mode not only results in higher productivity but also aids in the cost-effective process [32]. Obtained results also provide an opportunity for developing a cost-effective fermentation process where sucrose-rich renewable substrates could be used for levan production as a less expensive process. In addition, the fed-batch mode of fermentation has a high scope of improving levan productivity which is the need of the hour for industrial-scale production.

Results from GPC analysis indicate the appearance of high and low molecular weight fractions for 100 g/L initial sucrose concentration and upon increasing the sucrose concentration to 250 g/L, only low molecular weight fractions were observed. Raga-carbajal *et al.,* (2015) have investigated the mechanism of high and low molecular weight levan synthesis from *Bacillus* spp [33]. The non-processive synthesis of low molecular weight levan is mainly due to the oligosaccharide interaction with at least four acceptor binding sites. Raga-carbajal *et al.,* (2015) have also done a structural comparison of different levansucrase *Bacillaceae* families and found that differences in low molecular weight fractions synthesized are due to the structural changes in the upper receptor binding site [33]. However, the processive synthesis of levan with a high degree of polymerization remains unclear to date. A secondary site for oligosaccharide binding site in the vicinity of the catalytic pocket might be in the elongation of a high molecular weight fraction or could be responsible for the formation of branches during levan formation. The exact role of this ancillary site is currently under investigation [25]. When the initial sucrose concentration was increased to 100 and 250 g/L, a predominantly low molecular weight fraction was observed (Figure 4).

This type of levan-fructooligosaccharides (LFOS) could be used for various food applications. Fructooligosaccharide (FOS) consists of short chains of fructose with glucose at the terminal end. LFOS exhibits various health benefits for humans if ingested in appropriate quantities. They have low-calorie values and are non-carcinogenic in nature. FOS has been demonstrated to improve the absorption of Ca and Mg in the gastrointestinal tract, provide resistance to infections, bolster the immune system, and encourage overall nutrient utilization [34]. It has been reported that FOS kindles the growth and activity of gut bacteria, such as *Bifidobacteria* spp. and *Lactobacilli* spp. [35]. Studies conducted on FOS have indicated that FOS improves the memory and cognitive abilities of Alzheimer's patients [36] and helps to reduce blood plasma phospholipid, cholesterol, and triglyceride levels [37]. Hence, there is an increased demand for FOS production in the industry[38]. The production of LFOS by two-stage fed fermentation could be an efficient approach to maximize the production level of levan and LFOS for food industry applications.

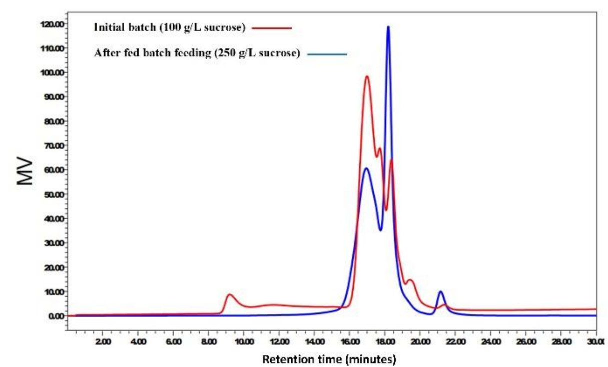

Fig. 4. Gel permeation chromatogram of levan produced from fed-batch fermentation.

3.3 Intrinsic viscosity and molecular conformation

Discussion of the concentration of levan solution (c) is impossible without knowledge of [η]. The intrinsic viscosity of the solutions also provides an idea of the fundamental molecular properties of the molecules in the solution. Based on the calculated coefficient of determination, the linear fittings were weighed (R^2). The most common models used to compute the [η] of polysaccharides are Huggins (Eq. (4)) and Kraemer (Eq. (5)). However, both models failed to predict the when fitting levan solution. Lack of application to solutions with concentrations below the critical value (c), which reduces the number of accessible experimental points, may be the cause of a poor fit. However, the better fit provided by the Tanglertpaibul Rao and Higiro models shows higher efficiency to determine intrinsic viscosity based on slope. Huggins and Kraemer's approaches were improved upon by this method, which is also unrestricted by the shear spectrum. This may be due to fewer experimental points available due to the model's restricted applicability to levan solutions with concentrations below the critical value (c). Therefore, the finding of the intrinsic viscosity from the slope of the dilute region seems to give a more reliable range even at different shear rates. The finding of [η] based on the slopes of the plots gives higher correlation coefficients and smaller standard errors than the intercept values of the plots [39-41]. Additionally, further dilution during sample preparation increases η_{sp}/C inaccuracy [42]. Literature shows that the Huggins equation is applicable only at $\eta_{sp} < 0.7$. In the present study, η_{sp} varied from 0.2 and 1.0.

Table 2. Intrinsic viscosity of the levan solutions.

Shear rate (s^{-1})	Tanglertpaibul Rao		Higiro-1		Higiro-2	
	R^2	η(dl/g)	R^2	η(dl/g)	R^2	η(dl/g)
132	0.9939	0.491	0.9915	0.3093	0.973	0.202
198	0.9788	0.473	0.9919	0.2972	0.998	0.198
264	0.9805	0.471	0.9928	0.2865	0.9991	0.1797

Table 3. The values of exponent b using power law and Berry number (C*η)

Shear rate (s^{-1})	R^2	b	Tanglertpaibul Rao (C*η)	Higiro-1 (C*η)	Higiro-2 (C*η)
132	0.9637	1.1514	0.1-1.22	0.077-0.77	0.051-0.505
198	0.9616	1.0227	0.12-1.2	0.07-0.74	0.05-0.49
264	0.9281	0.8584	0.12-1.18	0.07-0.72	0.04-0.45

The intrinsic viscosities determined by equations 5 to 7 at different shear rates are shown in Table 2. The [η] of the levan solution obtained from the Tanglertpaibul Rao plot is higher than the value from Higiro plots at different shear rates. Similar results were obtained when estimated for sage seed gum, Cress seed gum, and Xanthan gum [41, 43]. Even at higher concentrations of levan solutions, it showed low intrinsic viscosity, and also it exhibited a decrease in viscosity on increasing shear rate [44].

The plot of log (η_{sp}) vs. log (C[η]) was used to find the dilute Newtonian domain and the coil overlap parameter. The slope of the curve at a different rate and [η] from Tanglertpaibul Rao was in the range of 0.8 - 1.15 and all the samples were also in the dilute regime. The molecular entanglement of the levan solution at different concentrations can be observed using the Berry number (C[η]) if this dimensionless concentration exceeds one and the semi-dilute regime is determined to be in the range of 1.0-10.0. But in the current study, it was in the range 0.01- 1.2 for the concentration from 0.25 to 2.5 g/dl and believed to be free of coil overlap and molecule entanglement (Table 3). While the concentration between 5 to 10 g/dL exhibits a semi-diluted regime and is thought to be the result of molecule entanglement and coil overlap. The exponent b value derived from the power law equation (eq. 8) is shown in Table 3. The random coil conformation in a diluted regime is associated with the value $b > 1$, but the rod-like conformation is associated with $b < 1$. For levan solutions of different concentrations at different rates show the b value was in the range of 0.9 to 1.2 indicating that the molecular conformation of rod-like structure to random coil conformation upon changing shear rate. At a lower shear rate, it exhibited random coil conformation whereas at a higher shear rate exhibits a rod-like structure.

4 Conclusions

The current work investigated on levan production using different feeding strategies by *Bacillus subtilis* MTCC441 in batch and fed-batch modes of operation. A repeated feeding strategy on levan production resulted in an increased yield from 70 % to 96 % of the theoretical maximum on levan production. Scaling up of levan production from shake flask to bench scale fermenter was successfully implemented with increasing levan productivity from 1.75 g/L h (shake flask) to 4.38 g/L h (bioreactor) with 94 % of sucrose consumption within 8h of fermentation. On the fed-batch mode of fermentation, levan yield increased to 7.18 g/L h within 28 h. This is the first systematic study of this strain *Bacillus subtilis* MTCC441 in a fermenter that was reported with a higher yield. The results from this study help us to produce the levan on an industrial scale. The molecular weight distribution of levan depends on the initial sucrose of the medium. Feeding higher sucrose concentration in fed-batch fermentation, results in producing low molecular weight levan (4 and 10 kDa). This type of low molecular weight levan or fructooligosaccharides could be used as a prebiotic in food applications.

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