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Optimization of process parameters for levan batch fermentation by *Halomonas variabilis* MTCC 3712

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Abstract

The batch shake-flask production of bacterial levan from *Halomonas variabilis* (MTCC 3712) culture fermentation was investigated. Fermentation medium composed of sucrose and jaggery (a traditional concentrated sugar cane juice) as carbon substrates were used to study the maximum levan yield and it was found that the moderate concentration (300 g/L) of sucrose had yielded 10% increase in the levan production compared to high concentration (500 g/L). Replacement of Jaggery (for sucrose) in the medium gave an additional 10% increase in levan yield. Fermentation medium variables were screened using Plackett-Burman design and the three screened variables were analyzed for their variance in among them, by central composite design. Fourier Transform InfraRed (FTIR) spectroscopic analysis was done to confirm the functional groups of synthesized levan and compared with that of standard levan.

Keywords: ANOVA, FTIR, *Halomonas variabilis*, Jaggery, levan, PBD.

INTRODUCTION

Microbial extracellular polysaccharides (EPSs) are getting to be potential biomolecules that snatch the real segment of current biopolymer market. A recent review pointed out four EPSs, specifically, xanthan, pullulan, curdlan and levan as biopolymers with extraordinary applications in biopesticides, biofertilizers, feed additives in agrochemical segment, biopharmaceuticals and therapeutics in the healthcare sector and as biofuels in energy and environment sectors [1]. Levan, a branched homoexopolysaccharide, is composed of D-fructo-furanosyl groups linked each other by β – (2,6) linkages in the fundamental chain and by β – (2,1) joins at the branches. Levan offers incredible advantages for bacterial strains which produce it, includes bacterial survival in soil, phytopathogenesis and mutualism. Levan has numerous properties like chemical and water holding capacity, viscosity, film formability, capacity to dissolves in oil and water, chemical resistance and heat stability, which draws it as an extraordinary biopolymer [2].

Levan was secreted extracellularly from sucrose-based substrates by wide variety of bacteria, including genera *Acetobacter, Bacillus, Erwinia, Gluconobacter, Halomonas, Microbacterium, Pseudomonas, Streptococcus, Zymomonas*, etc [1]. Each of these bacteria have utilized different (synthetic) sucrose and (natural) sucrose-containing substrates for their growth, levansucrase synthesis and produced varied levan yields. Jaggery is known as agri-industrial residue, a conventional non-centrifuged, unpurified and concentrated sugar cane product, containing 75-80% sucrose was found to progressively viable substrate for some exopolysaccharides [3, 4, and 5].

In this current study, levan production using sucrose and jaggery as carbon substrates by *Halomonas variabilis* MTCC 3712, in batch shake-flask fermentations, was attempted. Further, the medium components and environmental factors were optimized using Plackett-Burman design and Central composite design. A comparison of structural characterization, using FTIR, for synthesized levan with standard levan was also done.

MATERIALS AND METHODS

Microorganism and culture development

Halomonas variabilis MTCC 3712 used in this research was procured from Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh, India. The strain was maintained on agar slants at 4°C and revived monthly on fresh medium. All the chemicals (analytical grade) used in this study were purchased from M/s. Merck India Ltd. Jaggery was purchased from local market and found to be mainly compose of sucrose (app. 75% w/w). A loop-full of freshly grown culture from agar slants were transferred to test tubes having growth medium (after autoclaving at 121°C, for 15 min.). The composition of growth medium used was as follows (in g/L): sucrose, 50; yeast extract, 2.5; K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.0, $MgSO_4{\cdot}7H_2O$, 0.5 (pH 5.0 ± 0.2).

Batch shake-flask fermentation experiments

The production medium used had the same composition of growth medium with an initial sucrose concentration (g/L) of 300 and 500 in batch shake-flask fermentations. An alternative carbon substrate (jaggery) was substituted with sucrose (300 g/L) on equal base to determine the effect of carbon substrate on levan production. Both sucrose and jaggery containing media in 100 ml aliquots were distributed in 250 ml Erlenmeyer flasks and autoclaved. These sterilized media were inoculated, 5% (v/v) aseptically and incubated for 168 hours at 37°C and 130 rpm on a rotary shaker. Fermentation broth samples were collected, aseptically, at irregular intervals and the concentrations of dry cell biomass, levan and residual sucrose were determined. Further, the statistical optimization studies were done by RSM using Plackett-Burman design and Central Composite design to evaluate the influence of process variables in levan synthesis.

Estimation of dry cell biomass, levan and residual sucrose concentrations

During fermentation, at specific intervals of time, 2 ml of broth volume from each flask was collected and centrifuged (13,000 rpm for 10 min.) The cell biomass (pellet) fraction was washed twice with saline water and dry cell biomass

weight (in g/L), after drying in an oven at 90^0C, was noted. The polysaccharide, levan, was precipitated (kept at 4^0C for 12 hours) using cell-free supernatant liquid by adding cold ethanol (in the ratio of 1:2 v/v). The content of levan (g/L) was measured after filtering the precipitate through Whatman No.1 filter paper and drying at 80^0C. The residual sucrose content in the cell-free fermentation broth was measured according to the Miller's [6] method using sucrose as the standard with Eppendorf (AG 22331) Biospectrophotometer. The data reported are the average values ± SD of three replicate experiments.

Levan characterization by Fourier Transform-IR spectroscopy

The precipitated levan from each flask, at the end of fermentation was characterized to compare the quality of levan obtained from sucrose and *jaggery* media. The structural characterization of levan was carried out using Fourier Transform InfraRed (FTIR) spectroscopy and IR spectra were recorded with FTIR Spectrophotometer, Tensor 27, Bruker optics, Germany.

Optimization of levan production by Plackett-Burman design

Seven variables showing the production medium components and fermentation conditions were tested at low (*L*) and high (*H*) levels with a total of eight runs were performed according to the Plackett-Burman (PB) design as shown in Table 1. All the runs were performed in triplicates using 250 ml Erlenmeyer flasks and the data analysis was reported (in Table 3) as the mean of triplicate.

Optimization of levan production by Central Composite design

For the selection of optimum conditions in case of multivariable system, Response Surface Methodology (RSM) is known as useful experimental design strategy [7]. The Central Composite Design (CCD) of RSM with 3 factors and 3 levels, including 6 replicates, all the center points have used for a second order response surface having an imbedded factorial matrix with center and axial points around the center point allowed the curvature estimation. A full-factorial design that has a factorial point with a distance value (= 1.0) of center to design space, with 3 factors. Each factor was studied into 3 different levels (*-1*: Low, *0*: Middle, *+1*: High) and actual design indicated a set of 20 experiments which include 6 center and 14 non-center (6 axial and 8 factorial) points (Table 2). The statistical analysis of data was calculated using Design Expert® ver. 11.1.2.0 (State-Ease, Inc.).

RESULTS AND DISCUSSION

Effect of initial sucrose concentration on levan production in batch culture

Many researchers have attempted different native bacteria to synthesize levan from sucrose as carbon substrate [1]. In general, high sucrose concentration favoured the transfructosylation reaction to produce levan [8]. *Halomonas variabilis* (MTCC 3712) was used for levan production in synthetic medium containing 300 and 500 g/L of initial sucrose concentrations. Submerged batch fermentations were carried out in 500 ml flasks with 100 ml working volume in an orbital incubating shaker at 37^oC and 130 rpm. *H. variabilis* (MTCC 3712) produced a large quantity of extracellular levan in 30% (w/v) sucrose containing production medium. The formation of levan was appeared as a cloudy after ethanol precipitation. Figure 1 shows the time profiles of sucrose utilization, biomass (dry weight) growth and levan formation for 300 g/L sucrose fermentation. A maximum concentration of 56.44 ± 0.54 g/L levan was produced after 168 hrs. [9] Also produced 48.9 g/L of levan from 20% sucrose medium using *Halomonas smyrnensis* AAD6. As expected, the residual sucrose concentration decreased during fermentation time, coinciding with an increasing levan and biomass concentrations. Culture conditions necessary for both the bacterial growth and levan production is unique for each bacterium.

Figure 2 shows the time course profiles of sucrose, biomass and levan using 500 g/L of initial sucrose concentration. In this, a highest 51.35 ± 0.38 g/L of levan was produced at 168 hours of fermentation, as cells entered the stationary phase (data not shown). With this increased sucrose concentration, levan formation was slightly reduced may be since the transfructosylation reaction held a prominent position and sucrose hydrolysis was significantly inhibited by high concentration of sucrose [10].

Study of jaggery as a carbon substrate on growth of H. variabilis and levan synthesis

The substitution of Jaggery (for sucrose) in the cultivation media with agar in petri plates was attempted to study the growth of *H. variabilis*, to investigate this agri-industrial residue as carbon substrate for levan synthesis. Till date, no research work was carried out using Jaggery as carbon source for levan production. In our earlier study, the replacement of Jaggery for sucrose was investigated for pullulan production [5]. So, in the present study an initial concentration of jaggery, 300 g/L was used in the production medium to determine the maximum levan concentration using *H. variabilis* MTCC 3712. Figure 3 depicts the profiles of sucrose utilization (from jaggery), biomass growth and levan production and a highest concentration of 62.12 g/L was obtained.

Statistical analysis by Plackett-Burman design

Seven fermentation variables (5 medium components and 2 conditions) were examined using Plackett-Burman statistical experimental design. This design was not only used to find the optimum combination of variables that gave the maximum levan yield and was also used to determine the most potential variables using very less experimental runs. The effect of seven variables was evaluated using 8 experimental runs. The mean effect, factor mean square (Variance effect) and *F*-test values were determined and reported in Table 3. Among all the seven variables evaluated, sucrose, yeast extract, and time were found to be more significant factors (*F*-test values: 94.82, 31.70 and 47.61, respectively), affecting the growth of *H. variabilis* and levan production, and were used for further optimization studies.

There have been very few studies reported on the use of Plackett-Burman design to screen the significant medium components and environmental factors for the levan production. Jathore, N.R. et al., 2012 [11] have reported that sucrose, ammonium chloride, sodium nitrate and casein peptone have significantly affected the levan production by *Pseudomonas fluorescens*. In another study, sucrose, zinc sulphate and manganese sulphate were identified as the most promising factors in levansucrase production by *Bacillus subtilis* [12]. Plackett-Burman design was also attempted to study the influence of sucrose, yeast extract and agitation rate as key variables on extracellular release of levan from *Glucanobacter nephelli* [13].

Table 1. Factors with coded levels examined in PB design experiment

Run No.	A	B	C	D	E	F	G	Response
	Sucrose (g/L)	Yeast Extract (g/L)	K_2HPO_4 (g/L)	$(NH_4)_2SO_4$ (g/L)	$MgSO_4.7H_2O$ (g/L)	pH	Time (hrs.)	Levan Yield (g/L)
1	200 (*L*)	2.5 (*H*)	1.0 (*H*)	0.2 (*L*)	0.5 (*L*)	9.0 (*H*)	120 (*L*)	16.615
2	400 (*H*)	0.5 (*L*)	0.2 (*L*)	0.2 (*L*)	0.5 (*L*)	9.0 (*H*)	144 (*H*)	52.4
3	200 (*L*)	0.5 (*L*)	0.2 (*L*)	1.0 (*H*)	1.0 (*H*)	9.0 (*H*)	120 (*L*)	22.115
4	400 (*H*)	0.5 (*L*)	1.0 (*H*)	0.2 (*L*)	1.0 (*H*)	7.0 (*L*)	120 (*L*)	39.06
5	400 (*H*)	2.5 (*H*)	0.2 (*L*)	1.0 (*H*)	0.5 (*L*)	7.0 (*L*)	120 (*L*)	31.7
6	200 (*L*)	0.5 (*L*)	1.0 (*H*)	1.0 (*H*)	0.5 (*L*)	7.0 (*L*)	144 (*H*)	39.77
7	400 (*H*)	2.5 (*H*)	1.0 (*H*)	1.0 (*H*)	1.0 (*H*)	9.0 (*H*)	144 (*H*)	44.56
8	200 (*L*)	2.5 (*H*)	0.2 (*L*)	0.2 (*L*)	1.0 (*H*)	7.0 (*L*)	144 (*H*)	21.06

(*L*) – Low; (*H*) – High

Table 2. Experimental design used in CCD studies

Run No.	*A*	*B*	*C*	Response	
	Sucrose concentration (g/L)	Yeast Extract concentration (g/L)	Time (hrs.)	Observed Levan (g/L)	Predicted Levan (g/L)
1	300 (*0*)	1.5 (*0*)	132 (*0*)	62.5±0.36¤	62.15
2	300 (*0*)	1.5 (*0*)	152.18 (*++1*)	26.43±4.43	32.51
3	400 (*+1*)	2.5 (*+1*)	120 (*-1*)	117.12±3.12	114.11
4	300 (*0*)	-0.182 (*--1*)	132 (*0*)	14.73±2.64	12.91
5	300 (*0*)	1.5 (*0*)	111.81 (*--1*)	64.08±3.21	60.84
6	300 (*0*)	1.5 (*0*)	132 (*0*)	61.2±1.12	62.15
7	200 (*-1*)	2.5 (*+1*)	144 (*+1*)	11.38±3.21	8.15
8	468.18 (*++1*)	1.5 (*0*)	132 (*0*)	89.39±5.23	95.21
9	300 (0)	1.5 (*0*)	132 (*0*)	61.9±1.12	62.15
10	131.8 (*--1*)	1.5 (*0*)	132 (*0*)	23.5±3.12	20.51
11	300 (*0*)	1.5 (*0*)	132 (*0*)	62.22±0.03	62.15
12	300 (*0*)	1.5 (*0*)	132 (*0*)	62.3±0.03	62.15
13	400 (*+1*)	0.5 (*-1*)	120 (*-1*)	47.26±1.02	48.46
14	200 (*-1*)	2.5 (*+1*)	120 (*-1*)	5.34±1.78	7.53
15	200 (*-1*)	0.5 (*-1*)	120 (*-1*)	36.12±3.12	39.53
16	300 (*0*)	1.5 (*0*)	132 (*0*)	63.3±0.53	62.15
17	200 (*-1*)	0.5 (*-1*)	144 (*+1*)	30.89±1.32	31.88
18	400 (*+1*)	0.5 (*-1*)	144 (*+1*)	18.35±3.43	14.14
19	400 (*+1*)	2.5 (*+1*)	144 (*+1*)	93.5±6.26	88.06
20	300 (*0*)	3.182 (*++1*)	132 (*0*)	43.5±4.62	48.16

¤Mean ± Standard Deviation from three replicate experiments

Table 3. Statistical analysis of Plackett-Burman design of each variable at different levels for levan production by *H. variabilis*.

Variable Code	Component/ conditions	*H*	*L*	Mean Effect	Factor Mean Square	*F*-Test
A	Sucrose, g/L	200	400	17.04	580.72	94.82
B	Yeast Extract, g/L	0.5	2.5	-9.8525	194.14	31.70
C	K_2HPO_4, g/L	0.2	1.0	3.1825	20.25	3.30
D	$(NH_4)_2SO_4$, g/L	0.2	1.0	2.2525	10.14	--
E	$MgSO_4.7H_2O$, g/L	0.5	1.0	-3.4225	23.42	3.82
F	pH	7.0	9.0	1.025	2.10	--
G	Time, hrs.	120	144	12.075	291.61	47.61

D, F: Dummy variables

Figure 1. Time course for batch fermentation of *H. variabilis* MTCC 3712 in 300 g/L initial sucrose concentration. (—■—-Sucrose, —●—-Biomass, - ♦ -Levan)

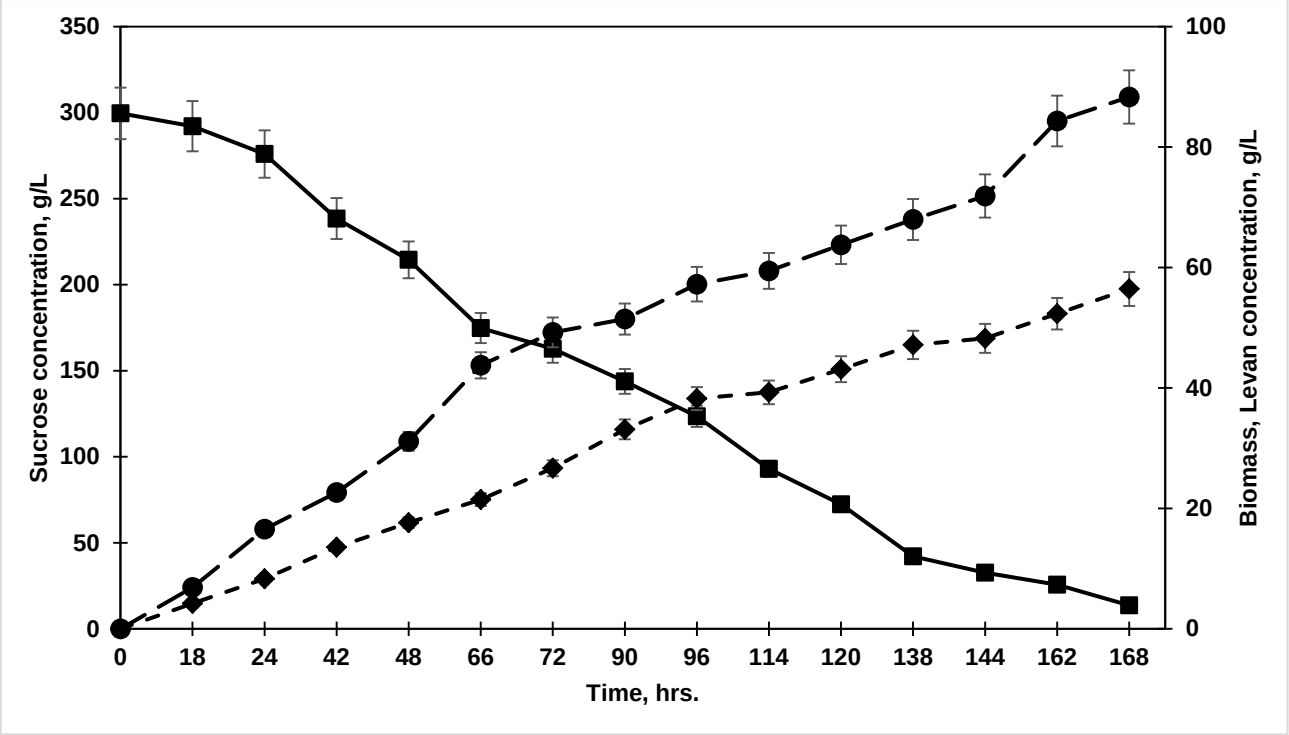


Figure 2. Time course for batch fermentation of *H. variabilis* MTCC 3712 in 500 g/L initial sucrose concentration. (—■—-Sucrose, —●—-Biomass, - ♦ -Levan)

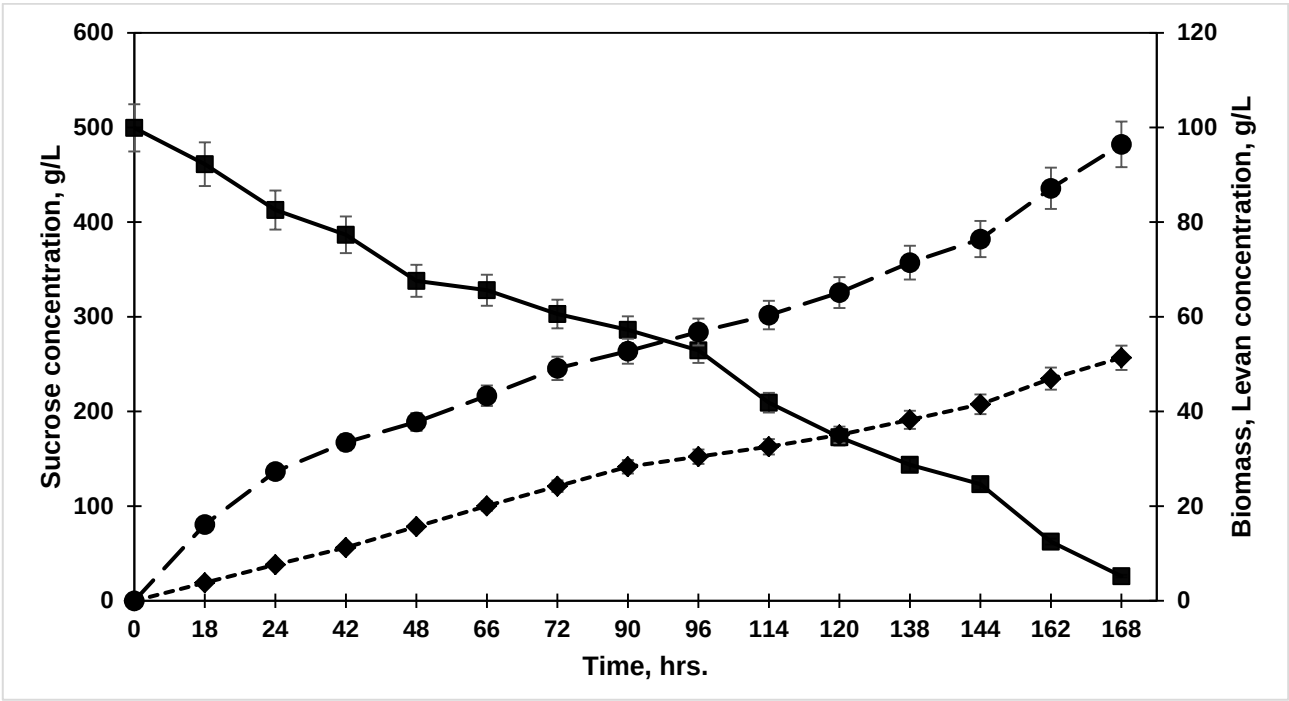


Figure 3. Time course for batch fermentation of *H. variabilis* MTCC 3712 in 300 g/L initial sucrose (jaggery) concentration. (—■—-Sucrose, —●—Biomass, - ◆ -Levan)

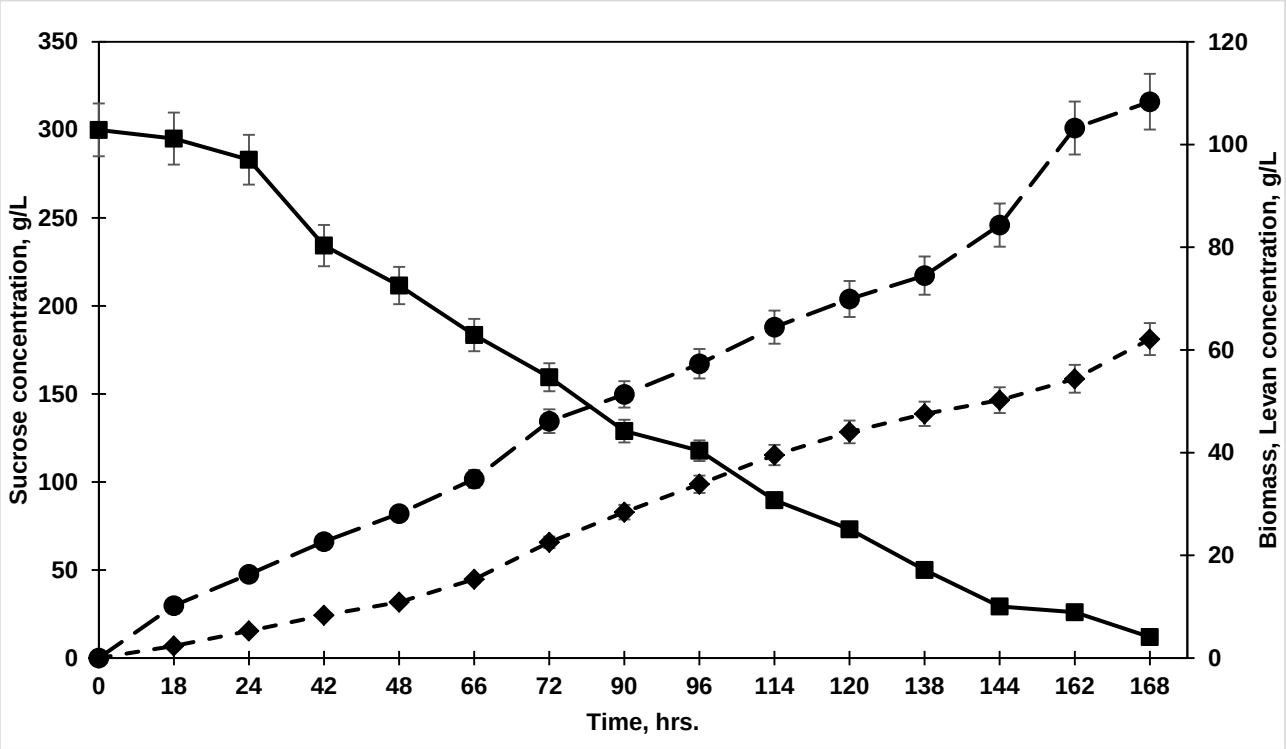


Figure 4. (a) *Pareto* plot showing the relation between actual and predicted values for levan maximization, 3-Dimenstional Response surface plots for levan production using (b) *A*: Sucrose – *B*: Yeast Extract interaction, (c) *A*: Sucrose – *C*: Time interaction and (d) *B*: Yeast Extract – *C*: Time interaction.

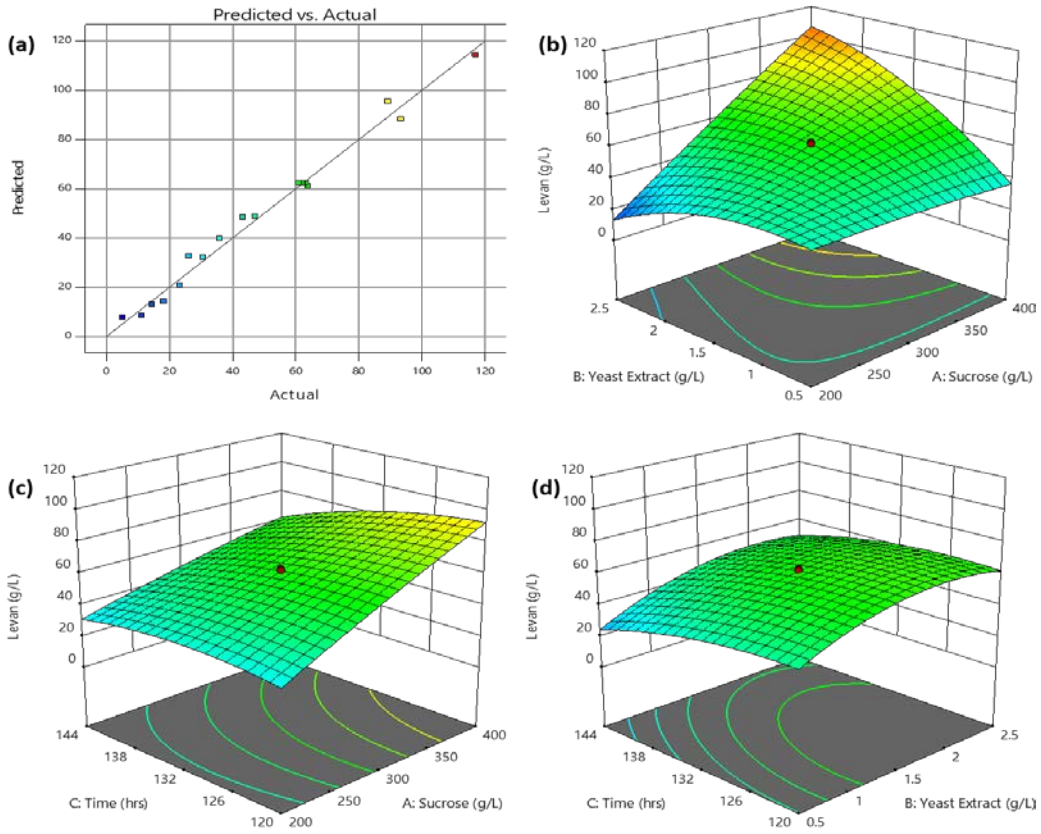
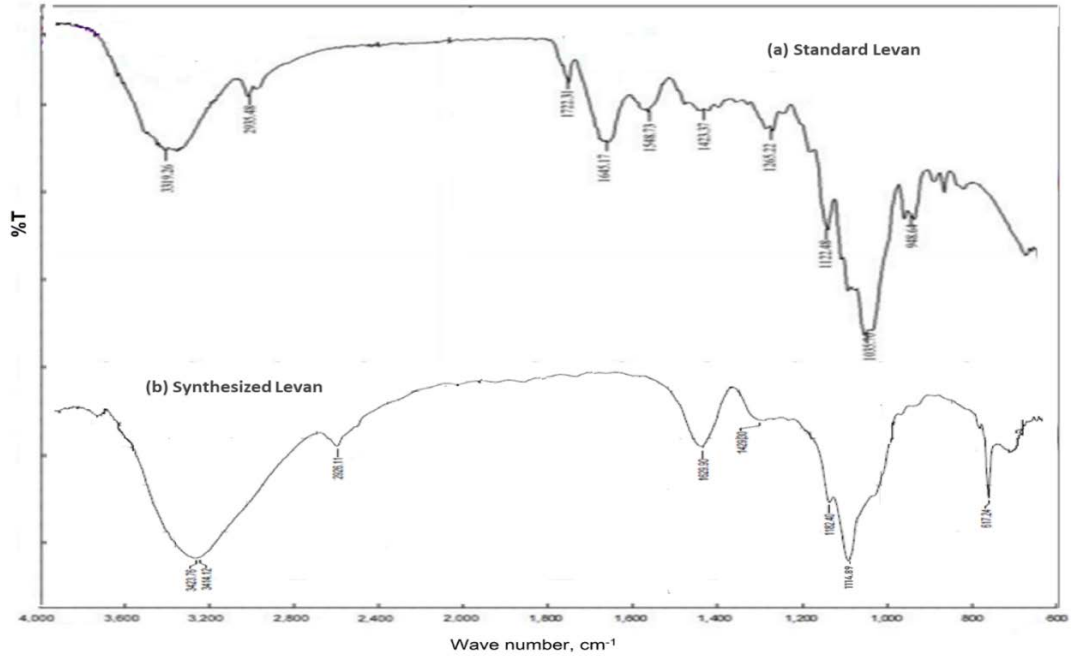


Figure 5. FTIR spectrum of (a) standard levan [15], (b) levan synthesised in this work.



Statistical analysis by Central Composite design

The use of Central composite design was a successful tool in the determination of optimal level of fermentation conditions with their interaction [7]. PB design of this study resulted the variables, sucrose, yeast extract and fermentation time were most significant factors (based on F-test values) in levan production. So, we performed a total of 20 experiments with varied combinations of initial sucrose concentration (g/L), yeast extract concentration (g/L) and time (hrs.) for levan production, as per Table 2. Fisher's statistical test for analysis of variance (ANOVA) was done to perform the statistical analysis. ANOVA predicted a quadratic model that the data and the model was significant ('*Prob>F*' less than 0.0001) for desired response (levan concentration) and confirmed the more accuracy of model. A second order polynomial equation for levan production, *Y*, in terms of coded factors was expressed as:

$$Y = +62.16 +22.21A +10.48B -8.42C +24.41AB -6.67AC +2.07BC-1.52A^2 -11.18B^2 -5.47C^2$$

The determination coefficient, R^2, shows the statistical significance of quadratic model (which should be closer to 1), indicates aptness (strength) of the model [14]. In this study, obtained R^2 value of model was 0.9878, the adjusted and predicted R^2 values were 0.9768 and 0.9077, respectively, also confirmed that this model is expected to predict the response (as the difference is < 0.2) more accurately. Further, signal to noise ratio of 33.426 (> 4 is desirable) indicates an adequate signal and thus model can be used to navigate the design space. The optimum of location (as per differentiation of quadratic model) for yielding maximum levan production was with A = 347.872 g/L, B = 2.312 g/L and C = 120.194 hrs, with desirability of 1.0. The *Pareto* plot obtained in diagnostics, showed more satisfactory correlation between the experimental and predicted values, wherein, more points clustered on and around the diagonal line, validates the goodness of model fit (Fig. 4(a)).

The 3D response surface plots show the graphical representations of regression equation by analysing the interaction between all the three factors with all possible combinations and visualizes the relation between the response and experimental level of each variable to estimate the optimum level of each factor required for maximum production of levan by *Halomonas variabilis* MTCC 3712. Figure 4 (b), (c), (d) portray the interactions between each pair (*AB*, *AC*, and *BC*) of factor at a specific factor (*C*, *B*, and *A*) and their effect, respectively, on levan elaboration. Fig. 4 (b) showed that strong interaction between initial concentrations of sucrose and yeast extract and low initial concentration of sucrose yielded low levan concentration, whereas it increases gradually when the sucrose concentration is increased in the medium. Initial sucrose concentration (300 g/L) and yeast extract concentration (1.5 g/L) indicated the maximum levan concentration of 63.31 g/L. In an earlier report, Haaland, P.D., 1989 (14) had demonstrated statistical (CCD) optimized batch production of levan using sucrose rich medium by *Acetobacter xylinum* NCIM 2526. Fig.4 (c) and (d) indicated that there was a strong interaction existed, between the factor sucrose – time and yeast extract – time, in optimal production of levan as the contour is elliptical. Levan concentration was reduced at low and high level and increased towards the middle level of sucrose and yeast extract.

Structural characterization of levan by FTIR spectroscopy

Structural characterization to examine the possible functional groups for synthesized levan from batch cultivation was done by FTIR Spectrophotometer. Figure 5 shows, the absorption peaks of standard levan (top spectrum) [15] and synthesized levan (bottom spectrum) from this study. Bottom spectrum indicating a characteristic

broad (strong) stretching absorption peak of O-H around 3,414.12 cm^{-1}, weak C-H vibration were seen at 2,926.11 cm^{-1}, carbonyl (C=O) stretching noticed at 1,629.9 cm^{-1}, and C-H stretching 1,182. cm^{-1}. In the specific area (950 - 1,200 cm^{-1}) is a characteristic of pyranose form of sugars, which allows the identification of major chemical groups in polysaccharide. The similar spectra and frequencies were also observed in other works [15, 16, and 17].

CONCLUSION

This work outlined the levan production by *H. variabilis* MTCC 3712 using sucrose and jaggery as carbon substrates. A levan concentration of 56.44 and 51.35 g/L was obtained from 300 and 500 g/L of initial sucrose concentration in production medium; whereas the levan yield was increased to 62.12 g/L by replacing sucrose with jaggery (300 g/L). This is the first attempt to study the utilization of jaggery as natural carbon (sucrose) substrate for producing levan in batch mode. Further, the medium components including fermentation conditions were also optimized using Plackett-Burman design and found that sucrose, yeast extract and fermentation time have shown significantly high *F*-test values. With these 3 factors, a central composite design was attempted to study the interactions among these variables in maximizing the levan production. An increased concentration (63.31 g/L) of levan was yielded with 347.872 g/L of sucrose concentration, 2.312 g/L of yeast extract concentration and 120.194 hrs of fermentation time. In addition, structural elucidation of functional groups in levan structure was done by FTIR spectroscopy.

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