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Scaling up of levan yield in *Bacillus subtilis* M and cytotoxicity study on levan and its derivatives

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This study focused on kinetics of levan yield by *Bacillus subtilis* M, in a 150 L stirred tank bioreactor under controlled pH conditions. The optimized production medium was composed of (g/L): commercial sucrose 100.0, yeast extract 2.0, K_2HPO_4 3.0 and $MgSO_4 \cdot 7H_2O$ 0.2; an increase in both carbohydrates consumption and cell growth depended on increasing the size of the stirred tank bioreactor from 16 L to 150 L. The highest levansucrase production (63.4 U/mL) and levan yield of 47 g/L was obtained after 24 h. Also, the specific levan yield ($Y_{p/x}$) which reflects the cell productivity increased with the size increase of the stirred tank bioreactor and reached its maximum value of about 29.4 g/g cells. These results suggested that *B. subtilis* M could play an important role in levan yield on a large scale in the future. Chemical modifications of *B. subtilis* M crude levan (CL) into sulfated (SL), phosphorylated (PL), and carboxymethylated levans (CML) were done. The difference in CL structure and its derivatives was detected by FT-IR transmission spectrum. The cytotoxicity of CL and its derivatives were evaluated by HepGII, Mcf-7 and CaCo-2. In general most tested levans forms had no significant cytotoxicity effect. In fact, the carboxymethylated and phosphrylated forms had a lower anti-cancer effect than CL. On the other hand, SL had the highest cytotoxicity showing SL had a significant anti-cancer effect. The results of cytotoxicity and cell viability were statistically analyzed using three-way ANOVA.

[**Key words:** Levan; *Bacillus subtilis*; Fermentor; Chemical modification; Cytotoxicity]

Levan is a fascinating β-(2,6)-linked fructose polymer with a unique combination of properties. The backbone of this homopolysaccharide consists of D-fructofuranosyl residues. Levansucrase is one of the most important fructosyltransferase enzymes (sucrose: 2,6-β-D-fructan-2,6-β-D-fructosyltransferase, EC 2.4.1.10) responsible for β-(2 → 6) levan from the transfructosylation reaction (1,2).

Levan has many potentials uses as an emulsifier, stabilizer and thickener, encapsulating agent, osmoregulator, and cryo-protector in a number of pharmaceutical and chemical industries such as food and cosmetics. It is used to prolong drug activity, as a radio-protector, and as an antitumor and cancer preventive agent (3–6). A few years ago, levans were found to have antiviral and fibrinolytic activities (7,8). In the last two decades, there has been a great expansion in the study of levan as a multifunctional polysaccharide. Many microorganisms such as *Bacillus subtilis*, *Aerobacter levanicum*, *Erwinia herbicola*, *Streptococcus salivarius* and *Zymomonas mobilis* were found to yield high molecular weight levan when grown on sucrose medium (9). The biggest limitation of the levan polymer is the cost. It is relatively expensive in comparison to other polysaccharides on the market. Due to its high cost, levan has only been produced and utilized in small quantities. Multiple properties of levan gave it great importance in the industrial field, which drew researchers' attention to try producing large quantities (6).

Levansucrase EC 2.4.10 is considered one of the most important enzymes which attracted interest of researchers in the last few years. This catalyzes β-(2,6)-levan synthesis through sucrose hydrolysis to glucose and fructose (7).

Recently, levan derivatives have gained attention since they have the potential to increase its biological activity. Levan derivatives could be explored as promising bioactive compounds with antioxidant and antitumor properties. It was noticed that polysaccharide derivatives had stronger antioxidant or antitumor activities than their non-derivatized counterparts (5,9,10). Thus, chemical modifications of polysaccharides give an opportunity for developing new agents with possible therapeutic uses. A few years ago, it was reported that *Paenibacillus polymyxa* EJS-3 levan was successfully acetylated, phosphorylated and benzylated, affording three levan derivatives with potential anticancer and antioxidant activities (11). Scientists are looking for the factors putting the body at risk and the factors which could protect against cancer.

This study focused on scaling up of *B. subtilis* M levan production in a 150 L bioreactor. Chemical modification of crude levan (CL) by sulfation, phosphorylation and carboxymethylation was done. The

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modification process was confirmed by different analytical methods. Previously, *B. subtilis* M levan was characterized as a strong anti-cancer agent (6). Accordingly, this work tries to evaluate the cytotoxicity of CL and their derivatives, against human colon carcinoma cell line (Caco-2), liver hepatocellular carcinoma (HepG2) and human breast adenocarcinoma (Mcf-7). The results confirmed the CL safety and also showed that the carboxymethylation and phosphorylation methods reduce the CL cytotoxicity and the cell viability increases. On the other hand, the sulfation methods lowered the cell viability and recorded the highest toxicity to the cancer cells compared to the other three levans forms. The statistical methods confirmed the validity of our results.

MATERIALS AND METHODS

Microorganism The strain used in this study was previously isolated from mountain-honey, a honey bee collecting nectar from desert flower grown in Libya. Honey was fresh non-treated ripe honey (directly collected from beehives). It was identified as *B. subtilis* M based on molecular identification (7). The strain was first cultivated on Luria Bertani (LB) agar medium of the following composition (g/L): tryptone, 10; yeast extract, 5; NaCl, 10; and agar, 20 (pH 7.0). After 24 h cultivation at 30 °C, the arisen colonies were harvested in 50% glycerol solution (v/v) and stored in 2 mL cryovials (Nalgene Nunc Int., Rochester, NY, USA) and stored at −80 °C as master cell bank to minimize intra-population and inoculum quality variations during this study.

Fermentation medium Firstly, *B. subtilis* M was cultured on LB broth medium. Then it was transferred to production medium with the following composition (g/l): yeast extract, 2.0, commercial sucrose, 100, $MgSO_4 \cdot 7H_2O$, 0.2 and K_2HPO_4, 3.0 (pH 7.0). Cultivation was conducted with 24 h vegetative cells and inoculum size of 5% v/v (inoculum density was measured at OD_{600}). The inoculum culture for the bioreactor was grown by inoculating 250 µL of *B. subtilis* M in a 250 mL shake flask (50 mL working volume) and incubated on a rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 200 rpm and 30 °C for 24 h. The pre-inoculum was inoculated in 4 L working volume of a 7 L stirred tank bioreactor for 24 h, then transferred to an 80 L working production medium of 150 L stirred tank bioreactor.

Bioreactor cultivation conditions Bottom drive stirred tank bioreactor 150 L (Bio Engineering, Wald, Switzerland) with working volume of 80 L, tank diameter (dt) = 410 mm and two 6-blade Rushton turbine impellers (impeller diameter (di) = 200 mm, di/dt = 0.488) was used. Aeration was performed using filtered sterile air and supplied continuously to the bioreactor with rate of 0.5 vvm. The agitation speed was adjusted to 100 rpm and kept constant during cultivation time. Foam was suppressed by silicone antifoam addition. pH and dissolved oxygen (DO) were determined throughout the cultivation process using a liquid filled pH electrode and a DO polarographic electrode, respectively. In the pH-controlled culture, the pH was adjusted to 7.0 by cascading the pH controller with acid/base peristaltic pumps connected with 4 M HCl or NaOH. During the fermentation, the medium was filtered and analyzed for the determination of levan, cell biomass, DO and pH every 2 h for 36 h.

Determination of cell dry weight Samples, in the form of 2 flasks of 50 mL of broth, or 10 mL of broth in the case of the bioreactor cultures, were taken at different times during the cell cultivation in pre-weighed centrifugation tubes (Falcon, USA) and centrifuged at 6000 rpm for 20 min. A small fraction of the supernatant was frozen at −20 °C for carbohydrate analysis, whereas the remaining centrifuged cells were washed twice using distilled water, followed by centrifugation. The centrifugal tubes were then dried to a constant weight at 60 °C to determine the dry cell weight.

Determination of molecular weight Different concentrations of CL were prepared, and the flow time of equal volumes for each concentration at 30 °C was determined in a U-shaped Ostwald viscometer. Flow time of the same volume of distilled water was also determined as the control. Thus, specific viscosity/C (gsp) was estimated.

Degree of polymerization Degree of polymerization (DP) was determined according to the following equation: DP = molecular weight (MW) of CL/MW of the monomers.

Total carbohydrates consumption The total carbohydrates were determined spectrophotometrically according to the method of Dubois et al. (12). The samples were heated with sulfuric acid to hydrolyze the polysaccharides and hydrate the monosaccharides to form furfural from pentoses and hydroxymethylfurfural from hexoses. The solutions of furfural and hydroxyfurfural were then treated with a phenol reagent to produce a colored compound and measured spectrophotometrically using a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, UK).

Levansucrase assay Levansucrase assays were performed according to the method of Yanase et al. (13). Culture filtrate (0.5 mL) was incubated with 1 mL 20% sucrose (Sigma) and 0.5 mL 0.1 M acetate buffer at pH 5.2, and incubated at 30 °C for 15 min. The decreasing amounts of sugars produced were measured by glucose oxidase kits.

Polymer yield and purification The levan producing organisms were cultivated on a defined medium as described above. After the early stage of the stationary phase, the culture was centrifuged at 3000 rpm to remove *B. subtilis* M cells. The culture filtrate was dialyzed against deionized water for 48 h with dialysis membrane (MWCO 14,000 Da, diameter 60 mm) to remove the unfermented sucrose and any fermentation products with low molecular weight. Levan was precipitated by using two volumes of ice-cold ethanol (99%). The dialysate was frozen with liquid nitrogen and freeze dried to afford CL.

Chromatographic analysis Acid hydrolysis was done using 0.1 N HCl in a boiling water bath for 1 h. The hydrolysis product was analyzed by descending paper-chromatography using Whatman No. 1 and a solvent system of *n*-butanol: acetone: water (4:5:1, v/v/v) and sprayed with aniline phthalate (14). The acid hydrolyzed product was identified by high-performance liquid chromatography (model Hewlett Packard 1050).

Levan modifications The carboxymethylation was carried out according to the modified method of Ismail et al. (15). In a 100 mL round bottom flask, 5 g levan was dissolved in 50 mL distilled water, and then 5 g of sodium monochloroacetate was added. The reaction mixture was stirred at 400 rpm for 6 h at 70 °C. Following cooling, neutralization was done with 90% acetic acid. The resultant solution was dialyzed against distilled water in Visking tubing (pore sizes typically range from 10 to 100 Å for 1–50 K molecular-weight cutoff membranes) for 72 h. The remnant was concentrated and lyophilized to give dried carboxymethylated levan (CML).

Phosphorylation of levan The phosphorylation of levan was performed according to the method reported by Zhang et al. (16) with some modifications. CL (5 g) was soaked in 200 mL of dimethyl formamide and 25 mL triethylamine. Then 12.5 mL phosphoric acid (PA) was added dropwise while stirring overnight at room temperature. The resultant solution was dialyzed against distilled water with a 14,000 Da molecular weight cutoff membrane for 72 h. The remnant was concentrated and lyophilized to give dried phosphorylated levan (PL).

Sulfation of levan This was achieved through adopting the method of Ragab et al. (17) by using sulfuric acid as the sulfation agent. Fuming H_2SO_4 (45 mL) was dropped in 80 mL frozen formamide in an ice bath. The reaction mixture was poured into 3 g of CL wetted by 5 mL of formamide. The reaction mixture was stirred overnight at 25 °C and then, diluted with 10 mL water. Neutralization was accomplished by adding 30% NaOH. Then dialysis as described previously was performed and the sample was dried to give sulfated levan (SL).

Physiochemical characterization The total carbohydrate content of levan and its derivatives were determined using fructose as the standard in the phenol-sulfuric method of Dubois et al. (12). The molecular weights of samples were measured at 30 °C in a U-shaped Ostwald viscometer. Flow time of the same volume of distilled water was also determined as the control. Thus, specific viscosity/C (gsp) was estimated. Purity of CML was confirmed by use of loss on drying, sodium and sodium chloride content according to Farm (18). The absolute value degree of substitution CML (DS_{CM}) was determined by the potentiometric titration method (19). The degree average of phosphorylation (DS_{PL}) was calculated by the method of Chen et al. (10). The degree of sulfation (DS_{SL}) was determined by the turbidimetric method of cleaving the sulfate ester groups. DS_{SL} was confirmed by the sulfur content of SL according to the method of Hussein and Helmy (20) as follows: An aqueous solution (4 mL) containing 100 mg of the investigated SL was titrated with an aqueous solution of toluidine blue. FT-IR spectra measurements were carried out using the KBr method. The infrared spectra of these samples were recorded with a Perkin–Elmer spectrometer (model spectrum BX) between 400 and 4000 cm^{-1} using a resolution of 4 cm^{-1} and 64 co-added scans.

Cell viability assay In the present study, the human hepatocellular carcinoma HepG2 cells, adenocarcinoma breast Mcf-7 cells, and colon carcinoma Caco-2 cells were used to evaluate the influence of chemical modifications of levan on its cytotoxicity on human solid tumor cells, compared with CL. The cells were grown in a 96-well plate in RPMI 1640 media (Bio Whittaker BE12-702F, Lonza, Basel, Switzerland) supplemented with 10 % fetal bovine serum (Gibco 10270) and antibiotics (streptomycin, penicillin-G, amphotericin B, Lonza). Cells (10^5 cells/mL) were incubated for 24 h at 37 °C in 5% CO_2 before being treated with different concentrations of levan derivatives and then incubated for 48 h at 37 °C. The cell viability was measured using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (21). This tetrazolium salt is metabolically reduced by viable cells to yield a blue formazan product measured at 540 nm spectrophotometrically. The mean of the cell viability values was compared to the control untreated cells and the percentage of cell viability was plotted against the derivative concentrations to calculate IC_{50}.

Statistical analysis Data was analyzed using a three-way model of analysis of variance (ANOVA) using Sigma Plot 12.5 software extended with the statistical package (22).

RESULTS AND DISCUSSION

Levansucrase production and levan yield in 150 L bioreactor Levan has high-value applications. Many strategies were done for the scaling up of levan and levansucrase. However, till this moment there were no sufficient studies to meet the industrial demand (7,23). Accordingly, one of our main goals in this study was scaling up of levan yield process using a 150 L fermentor and comparing its kinetics with our previous work (6). In this work, inoculum was prepared in a 16 L bioreactor (with working volume of 8 L). The results in Fig. 1 show that cells grew exponentially reaching 2.05 g/L after 22 h. During this phase, the levansucrase production and the levan yield (at controlled pH) recorded the highest value of 42.5 U/mL and 29.0 g/L, respectively, and the DO dropped in culture and reached almost 7 % saturation. The exponentially grown cells in the 16 L bioreactor were used to inoculate the 150 L bioreactor (with working volume of 80 L). As shown in Fig. 2, cells grew exponentially without lag phase in 150 L bioreactor, reached 1.60 g/L after 16 h and kept more or less constant for the rest of the cultivation time. Levan was produced in culture in parallel to the growth. It accumulated in the culture at a rate of 3.1 g/L/h, and reached a maximal value of levansucrase activity (63.4 U/mL) and levan yield (47.1 g/L) after only 24 h and kept more or less constant for the rest of cultivation time. The calculated levan should be not more than 39.5 g/L according to Ghaly's theoretical yield (24). The high value could be attributed to the production of another polymer or entrapped low molecular weight sugars. Also, the results pointed clearly to the firm correlation between the levansucrase production and levan yield, since there was a direct proportional between the enzyme production and levan yield as shown in Table 1. The majority of recent levansucrases studies aimed to the productivity of either the enzyme or the levan for commercial use (25). Wu et al. (26) examined *B. subtilis* (natto) Takahashi in 16 L fermentor for levan yield. The obtained levan value was 61 g/L in medium containing sucrose (250 g/L). This value was nearly matching Ghaly theoretical yield (24). In this study, during the polysaccharide production phase, sucrose concentration decreased gradually in the culture at a rate of 4 g/L/h and became limited in the culture after 24 h. Thus, the termination of levan yield and levansucrase production in this culture was due to carbon source limitation. During the active growth phase, the first 16 h of cultivation, the DO dropped

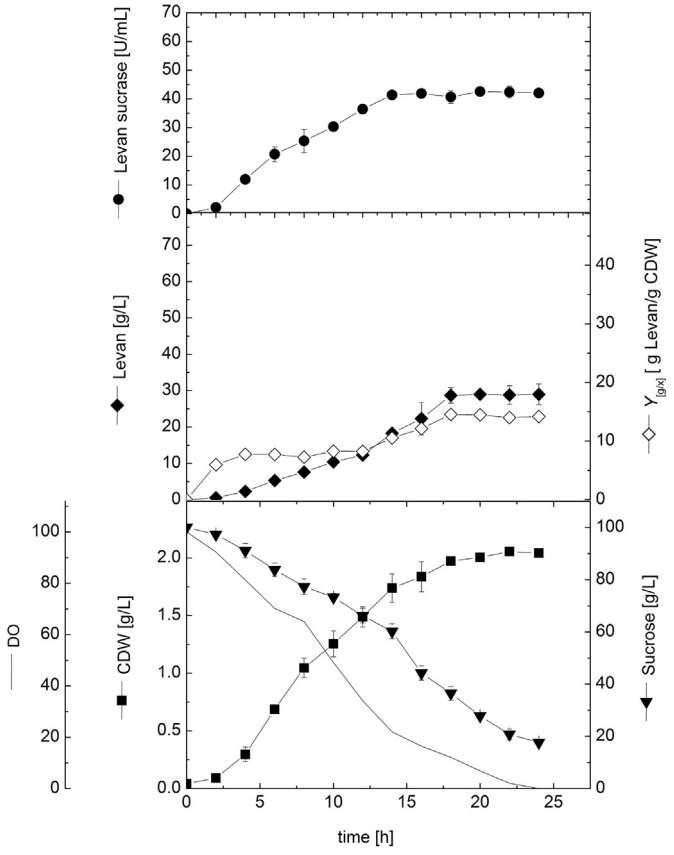

FIG. 1. Cell growth, levansucrase production and levan yield during submerged cultivation of *Bacillus subtilis* M in 16 L bioreactor for inoculum preparation stage.

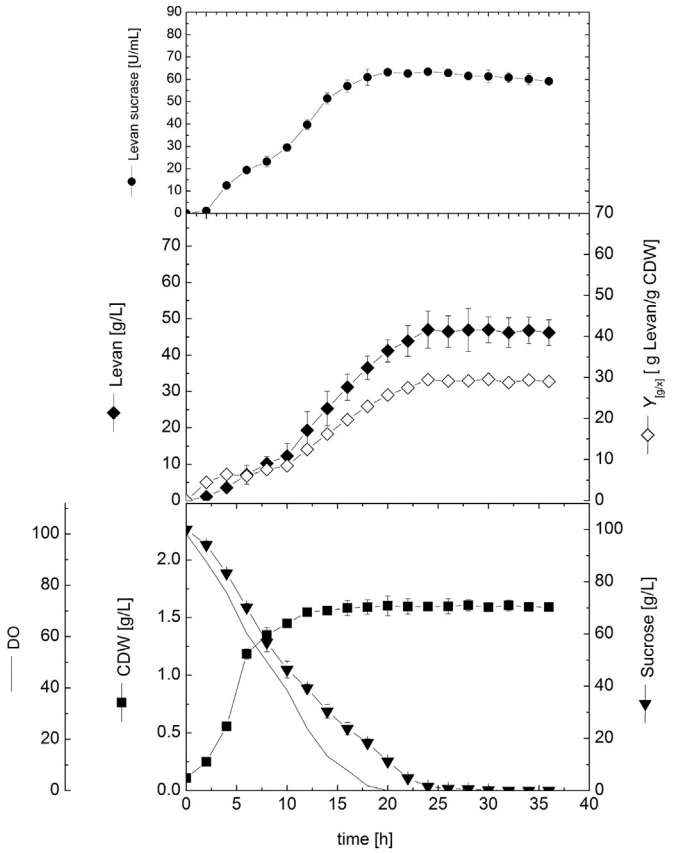

FIG. 2. Cell growth, levansucrase production and levan production during submerged cultivation of *Bacillus subtilis* M in 150 L bioreactor.

TABLE 1. Kinetics of cell growth and levan production by *Bacillus subtilis* M during batch cultivation in shake-flasks and in 16 L and 150 L stirred tank bioreactors.

Parameter	Shake-flask	Stirred tank bioreactor		
		Bioreactor 16 L		Bioreactor 150 L
		Uncontrolled pH	Controlled pH = 7	Controlled pH = 7
X_{max} (g/L)	1.60	1.70	2.05	1.60
dx/dt (g/L/h)	0.13	0.16	0.19	0.15
P_{max} (g/L)	18.60	22.10	29.00	47.10
$Y_{p/x}$ (g/g)	11.60	12.90	14.10	29.40
Levansucrase (U/mL)	21.1	33.8	42.50	63.4
t_f (h)	15.00	15.00	24.00	24.00
Mw (kDa)	35.50	–	50.00	72
DP	197.00	–	277.00	400.00

X_{max}, maximal dry cell weight; dx/dt, growth rate; P_{max}, maximal levan yield; $Y_{p/x}$, specific levan production (g levan/g biomass); t_f, fermentation time.

dramatically as function of fast growth and reached almost zero after only 18 h of cultivation. The obtained value of volumetric levan yield in this work is almost doubled of those value reported by other authors. Silbir et al. (27) showed that the maximum levan yield of 31.8 g/L was obtained using continuous cultivation at dilution rate of 0.14 h^{-1}.

Comparison of fermentation parameters The kinetics of levan yield by *B. subtilis* M, carbohydrate consumption and cell growth were previously studied in shake flasks and 16 L stirred tank bioreactor under controlled and uncontrolled pH conditions (6). Comparison between different growth parameters in the previous and current study was calculated and summarized in Table 1. The results revealed significant differences between levan yield and the cell growth kinetics when scaling up the process from 16 L to 150 L bioreactor. The maximal cell growth of 2.05 g/L was obtained at 16-L bioreactor, whereas the maximal biomass obtained in 150 L bioreactor was only 1.6 g/L. On the other hand, even the biomass in 150 L bioreactor was much less than 16 L, the levan yield increased from 29 up to 47.1 g/L. The result also pointed to the importance of pH control in increasing the levan yield. The same finding was observed by Ua-Arak et al. (28). It was reported in uncontrolled pH in fermentation process, the levan size/molar mass diminished continuously according to the continuous acidification of the nutrient medium. In terms of bioprocess development in scaling up, it is clear that scaling up the process from shake flask up to 150 L bioreactor resulted in a significant increase in the volumetric production by 2.5-fold. This high increase in production for the short time cultivation of only 24 h improved the economy process significantly with a high potential for levan production on an industrial scale. The molecular weights recorded 35.5, 50 and 72 kDa for flask, 16 L and 150 L bioreactors, respectively. This result insures that levan molecular weights were clearly differed according to the fermentation conditions variation (Table 1). On the other hand, the degree of polymerization was determined to be 197, 277 and 400 for levans from shake flask, 16 L, and 150 L bioreactors, respectively. In this finding, Franken et al. (25) mentioned that levan polymers mostly have a DP $>$ 100. On the contrary, it was reported that extracellular fructan from microorganisms had DP $<$ 100 (29). To our knowledge, no other research has been reported in scaling up of levansucrase production and levan yield up to 150 L bioreactor.

Levan modifications Levan was modified by carboxymethylation, phosphorylation and sulfation to three forms CML, PL and SL, respectively. The results obtained indicated that the yield was 83.5% CML, 79.8% PL, and 51.8% SL. The total carbohydrate content was determined for extracted levan to be 87.3%, and CML 79.2%, PL 82.7% and SL 63.9% for its derivatives. Acid hydrolysis of CL using 0.1 N HCl indicated high amounts of 98.5 % fructose and 1.5% glucose monosaccharide. Bekers et al. (30) also found monomers of glucose and fructose in levan after hydrolysis with hydrochloric acid and organic acids. The chromatograms of hydrolyzed levan by high performance anion-exchange liquid chromatography (HPAEC) showed two peaks, which were assigned to 21% glucose and 79% fructose (31). The sugar percent of derivatives were decreased after modification in comparison with levan due to substitution of different functional groups into levan reduced the fructose ratio. The average molecular weights of derivatives were slightly decreased compared to levan (35.5 KDa from shake flask). This could be due to partially degradation as shown in CML 31.3, PL 28.9 and SL 26.6 KDa. These results suggest that the polymeric nature was mainly composed by levan of low molar mass or fructo-oligosaccharides. The chain size of levan decreases by increasing concentration of sucrose; which explains the predominance of levan of low molecular weight in the initial times of the fermentation (30). The difference of molecular weights of crude *B. subtilis* M levan from 21 to 35.5 KDa is attributed to the change in environmental conditions (6,31).

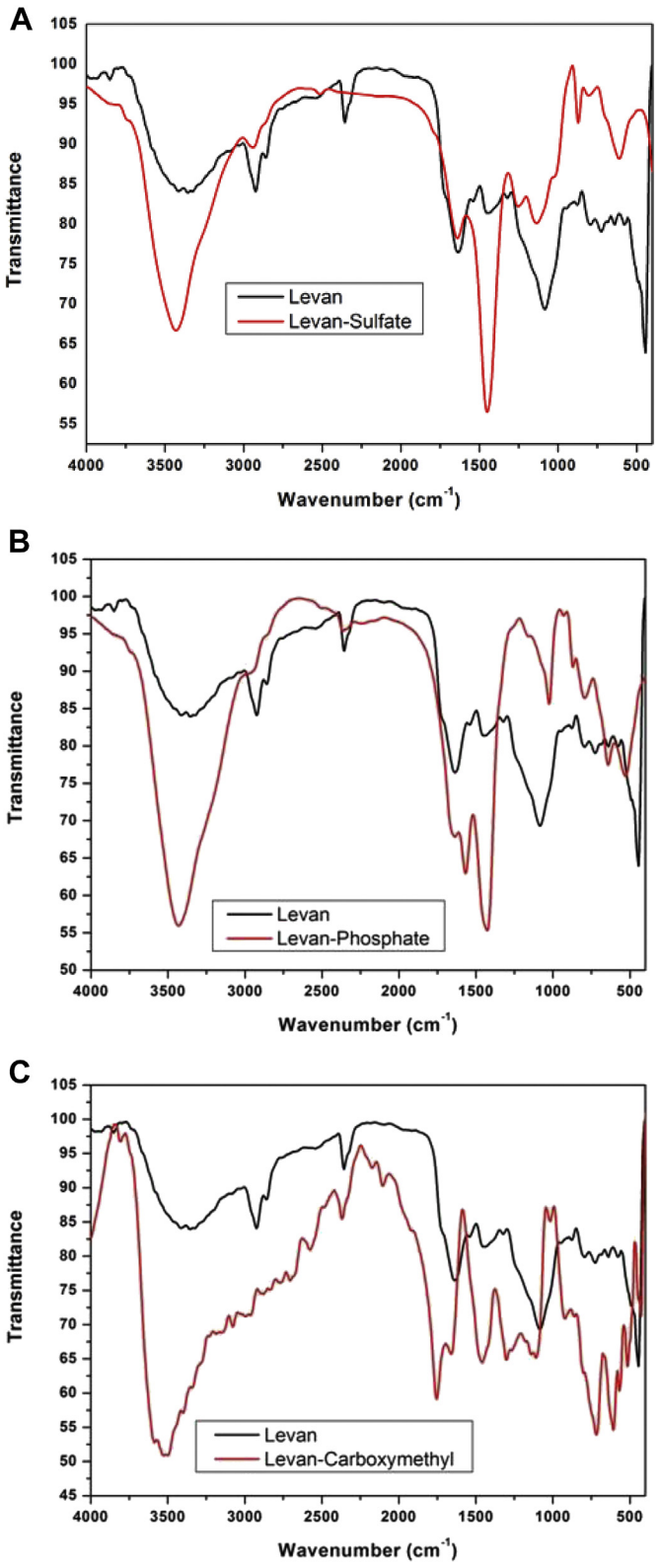

FIG. 3. FT-IR transmission spectrum of crude levan and its derivatives (A) CL and SL, (B) CL and PL and (C) CL and CML.

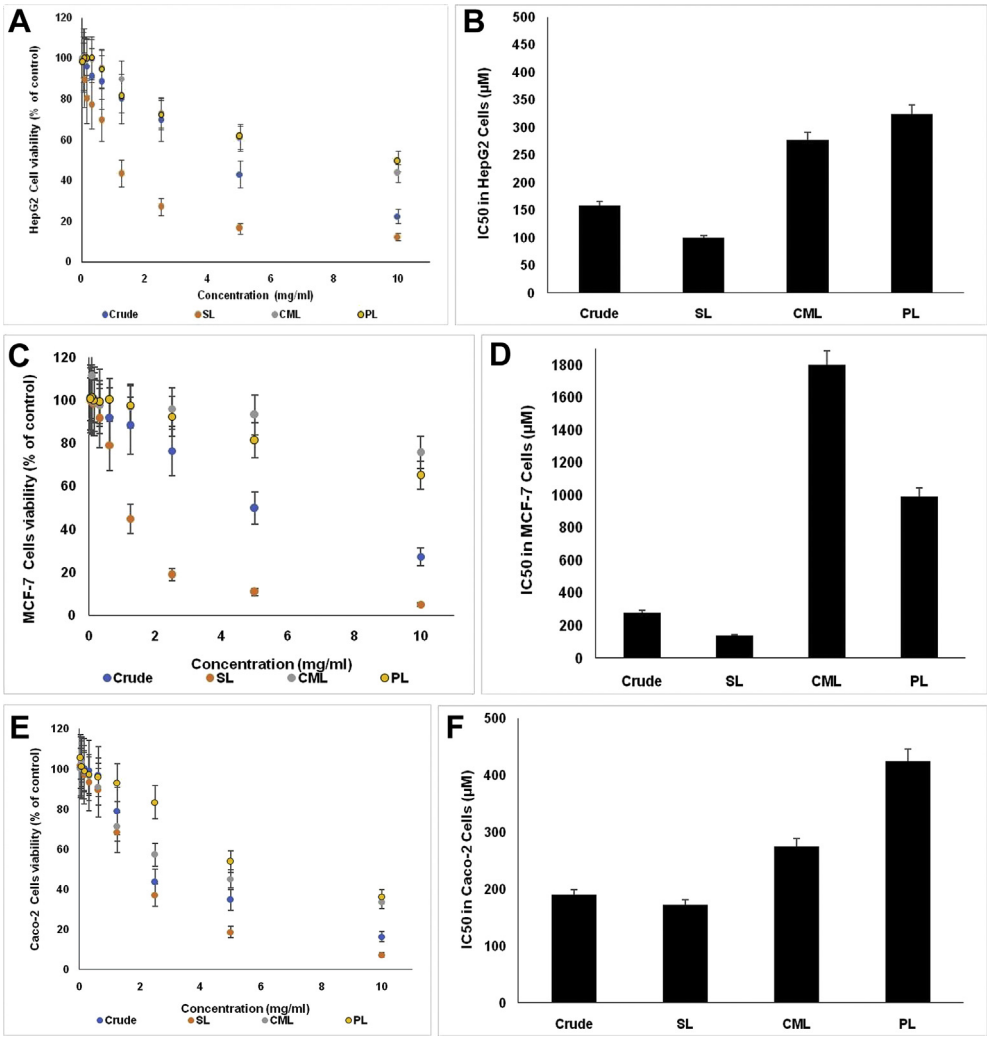

FIG. 4. The cytotoxicity of the crude levan and the chemically modified levan was investigated in human carcinoma HepG2 (A, B), Mcf-7 (C, D), and Caco-2 cells (E, F), using the MTT assay as a metabolic viability index. The cell viability results are expressed as the percentage of control cells (mean ± SE; A, C, E) and as the calculated IC_{50} (mg/ml; mean ± SE; B, D, F).

Levan was converted into CML by etherification and the process of carboxymethylation was optimized with respect to the ratio of sodium chloroacetate, temperature and time of reaction. Purity of CML was determined by many parameters: Loss on drying was 7% after drying. Also, the pH for 1 g of CML dissolved in 100 mL distilled water indicated neutral solution (pH 7.0–7.6). The total sodium content of CML was determined to be 9.3% by atomic absorption spectroscopy after completely drying. Sodium chloride content was 0.21%. These results revealed that CML has high purity. DS_{CML} increased with an increase in the sodium chloroacetate amount. This may be due to the greater availability of the acid molecules at higher concentrations in the proximity of the levan molecules. If sodium chloroacetate amount is low, the side reaction predominates with the formation of larger amounts of sodium glycolate, thereby lowering the degree of substitution of CML (DS_{CML}). The increase in DS_{CML} with temperature and time may be due to the fact that there is a better reaction environment created and a prolonged time available for carboxymethylation. The optimized CML product has DS_{CML} of 0.65. The highest degree of substitution could reach 1.22 (32,33). When the substitution degree of cellulose reached 0.85, the sample could be dissolved in water (34). During the carboxymethylation process, microwave irradiation can increase the degree of substitution of carboxymethyl and enhance the reaction between chloroacetic acid and polysaccharide (35).

The addition of sulfuric acid as the catalyst and the modification of polysaccharide with phosphoric acid is the first method of polysaccharide phosphorylation. The urea-phosphate method was commonly used, its temperature was 150 °C or so, and the molten urea was present, which was commonly used in the preparation of cellulose phosphate (36). After phosphorylation modification of levan, the branched hydroxyl group is substituted into the phosphate group. The phosphorous content and DS_{PL} were 2.41% and 0.13, respectively, and these values were higher than those reported by Liu et al. (11) (0.89% and 0.048) which determined according to Eq. 1.

$$DS_{PL} = 5.22 \times P\%/(1-2.61 \times P\%) \quad (1)$$

where P% is the phosphorous content.

Sulfation of levan is one of the most promising means for the production of value-added levan derivatives. The sulfation was optimized to gain a maximal concentration of sulfate groups. The principle of the experiment is as follows: the selected sulfuric acid reagent and levan are reacted under the required condition, and the functional groups on levan residues are modified with the sulfate

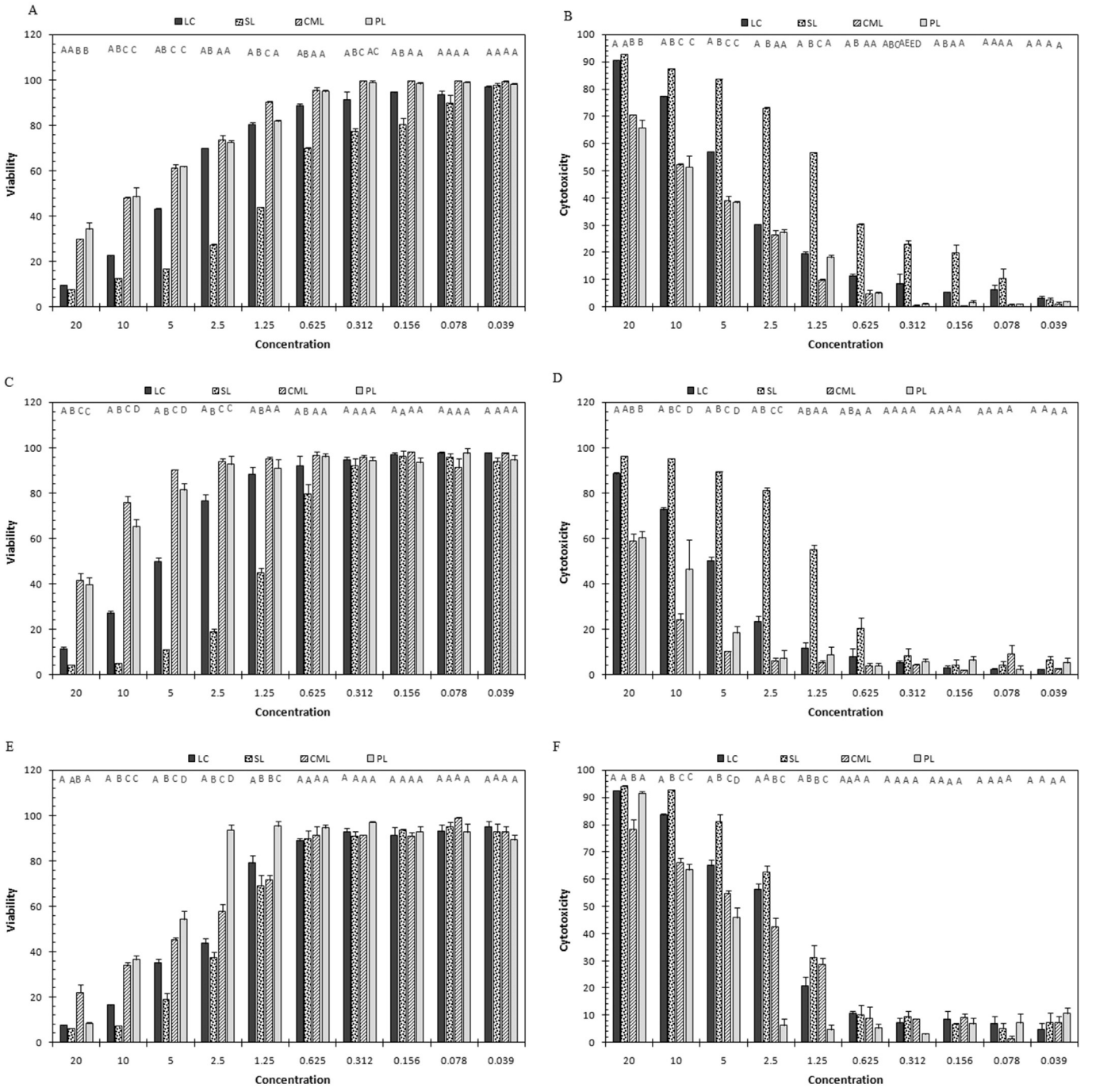

FIG. 5. The viability and cytotoxicity of the crude levan and the chemically modified levan against different cell lines. (A) HepG2 cell viability. (B) HepG2 cytotoxicity. (C) Mcf-7 cell viability. (D) Mcf-7 cytotoxicity. (E) CaCo-2 cell viability. (F) CaCo-2 cytotoxicity. Statistical analysis were done for the viability and cytotoxicity using three-way ANOVA.

anion. Investigated SL has the ability to react with 15.9 mL toluidine blue as a characteristic reagent for sulfated groups. The sulfate content was 17.8% and DS_{SL} was 2.1. DS_{SL} is higher than the value reported by Erginer et al. (37) (0.89) which determined according to Eqs. 2 and 3.

$$S\% = BaSO_4\ (\mu g) \times 0.1374 \times 100/\text{Sample}\ (\mu g) \quad (2)$$

$$DS_{SL} = 162 \times S\%/3200 - (102 \times S\%) \quad (3)$$

The DS values obtained by FT-IR spectra are the measurements of relative values of the degree of substitution. The difference in structure of levan and its derivatives was detected by FT-IR transmission spectrum as shown in Fig. 3A–C. The peaks at 3412 cm^{-1} and 3350 cm^{-1} represented O–H stretching of fructofuranose rings while the peaks at 2923 cm^{-1} and 2859 cm^{-1} correspond to C–H stretching vibrations of fructose rings. Sharp peak observed at 1100 cm^{-1} is due to C–O–C symmetric bending vibration of fructofuranose rings and glycosidic linkages (38). The FTIR transmission spectrum of SL is mentioned in Fig. 3A. The result showed that sulfation resulted in a slight blue shift of C–H stretching vibration from 2923 cm^{-1} to 2949 cm^{-1} with a decrease in its intensity as well as decease in the intensity of the peak which was previously noted at 2859 cm^{-1} in levan forming a small shoulder. There is also

a slight asymmetric stretching vibration of S=O linkages and symmetrical C–O–S vibration of C–O–SO_3 groups at 1250 cm^{-1} and 820 cm^{-1}, respectively (18). The FT-IR transmission spectrum of PL is shown in Fig. 3B. A remarkable decrease in the intensity of C–H stretching vibrations was clearly noticed in levan, as well as a slight band at 1300 which was assigned to the P=O stretching vibration, and redshift of the characteristic peak of 1083 cm^{-1} to 1025 cm^{-1} associated with a decrease in its intensity. FT-IR transmission spectrum of CML is shown in Fig. 3C. The peak at 1755 cm^{-1} corresponds to the carbonyl –C=O stretch of the protonated carboxylic acid group, and the weak shoulder at 1268 cm^{-1} is attributed to alcohol –C–OH stretch.

Cell viability assay In the present study, the cytotoxicity of CL and the chemically modified levan was investigated in human carcinoma cells HepG2, Mcf-7, and Caco-2, using the MTT assay as a metabolic viability index. In general, the results indicated the safety of CL and their derivatives with a degree of variation. In all the cells, lines used the carboxylation and phosphorylation methods lowered the CL cytotoxicity. The sulfation process clearly stimulated the cytotoxicity property. All results were divided in molecular weight. The results indicated that, in liver HepG2 cells, CL possessed a moderate cytotoxicity with IC_{50} of 157.99 μM while the carboxymethylation and the phosphorylation decreased this cytotoxicity, as can be concluded from the high IC_{50} 277.46 μM and 324.43 μM (Fig. 4A, B). On the other hand, the sulfation of levan increased its cytotoxicity in HepG2 cells (IC_{50} 99.39 μM), as shown in Fig. 3A, B. The cell viability findings in breast MCF-7 cells revealed that the treatment with CL resulted in a low cytotoxic effect with IC_{50} of 280.61 μM, whereas the carboxymethylation and the phosphorylation eliminated this effect and both of CML and PL were safe in MCF-7 cells and their IC_{50} were very high up to 1797.51 and 992.76 μM, respectively (Fig. 4C, D). However, the sulfation of levan elevated its cytotoxicity in MCF-7 cells (IC_{50} 137.09 μM; Fig. 4C, D). Using colon Caco-2 cells, the treatment with both of CL and SL led to a moderate cytotoxic effect with IC_{50} of 189.29 and 172.66 μM, respectively (Fig. 4E, F). On the other hand, both of CML and PL had weak cytotoxicity in Caco-2 cells and their IC_{50} were 274.47 and 423.94 μM, respectively (Fig. 4E, F). The previous results mentioned levan and its sulfated form as strong anticancer agents and SL possesses good anticancer activities (5). Liu et al. (11) reported acetylated levan, PL and benzylated levan had strong anticancer activity against human gastric cancer BGC-823 cells. In general, there was no significant cytotoxicity at low concentrations, and there was no promising loss in cell viability. There is a detectable cytotoxicity at high concentrations of CL and SL.

Statistical analysis The data concerning cytotoxicity and cell viability were statistically analyzed by three-way ANOVA test. The results in Fig. 5A–D confirmed the experimental results. Finally, this work is another trial to scaling up the *B. subtilis* M levan in 150 L fermentor. The results pointed to the success in obtaining levan efficiently in large-scale in a short time and using commercial sucrose as a low-cost material. This result confirmed the economic advantage for yield of *B. subtilis* M levan in 150 L fermentor. In addition, the results highlighted how the levan modification process could affect their properties from different aspects. Also, the results referred for the first time to the role of carboxylation and phosphorylation process in lowering CL toxicity to a great extent and the sulfation process in increasing CL cytotoxicity. The results confirmed the importance of CL and their derivatives to be applied in different pharmaceutical aspects.

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