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Scaling up, characterization of levan and its inhibitory role in carcinogenesis initiation stage‘

Mona A. Esawy[a,*], Hassan Amer[a,b,**], Amira M. Gamal-Eldeen[c], Hesham A. El Enshasy[d,e], Wafaa A. Helmy[a], Mona A.M. Abo-Zeid[f], Roslinda Malek[d], Eman F. Ahmed[a], Ghada E.A. Awad[a]

[a] Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Giza, Egypt
[b] Department für Chemie, UFT-Haus Universitäts- und For schungszentrumTulln (UFT), Konrad Lorenz Straße 24, 3430 Tullnan der Donau, Austria
[c] Cancer Biology Laboratory, Center of Excellence for Advanced Science, Department of Biochemistry, National Research Centre, Dokki, Giza, Egypt
[d] Institute of Bio-product Developments (IBD), Universiti Teknologi Malaysia (UTM), Skudai, Johor, Malaysia
[e] City for Scientific Research and Technology Applications (CSAT), Alexandria, Egypt
[f] Genetics and Cytology Department, Cancer Biology Laboratory, Center of Excellence for Advanced Science, National Research Center, Dokki 12622, Cairo, Egypt

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ABSTRACT

Honey isolate *Bacillus subtilis* M was cultivated in shake flasks and in 16-l bioreactor cultures to investigate cell growth, bio-metabolites production kinetics and bioprocess scalability. The respective maximal levan and levansucrase productions of 59.5 g/l and 74.1 U/ml were achieved in bioreactor cultures under pH controlled condition (pH = 7.0) after only 24 h. Crude levan (levE) was isolated, characterized and fractionated into F1, F2, and F3 with different molecular weight (21.8, 13.118, 9.53 kDa). ^{1}H NMR and ^{13}C NMR spectroscopy proved that LevE and their fractions were mainly β-(2, 6)-linked levan-type polysaccharide. The cancer chemo-preventive activity indicated that the levE and its fraction 3 were promising inhibitors of cytochrome P-450 1A activity, inducers of glutathione-S-transferase activity in Murine hepatomaHepa1c1c7cells and possessed highest radical scavenging affinity to both ROO$^{\bullet}$ and OH$^{\bullet}$. They inhibited the induced-DNA fragmentation. None of the tested samples triggered apoptosis or necrosis in splenocytes, except F2.

1. Introduction

Fructans are widely distributed as carbohydrate storage polymers in the vegetative tissue of many families of plants, bacteria, and fungi (Hosono et al., 2003). According to the type of linkage, fructans are classified into three families, namely, inulin [(2→1)-linked β-D-fructofuranosyl units], levan [(2→6)-linked β-D-fructofuranosyl units], and graminan [both (2→1)-linked and 2→6)-linked β-D-fructofuranosyl units] (Roberfroid, 2005). Microorganisms have been manipulated for the biotechnological production of polysaccharides with tailored properties suitable for high-value medical application such as cancer chemo-preventive properties (Gamal-Eldeen, Ahmed, & Abo-Zeid, 2009). Levan is a well-known polysaccharide its backbone consisted mainly of fructose. It has many potential uses as emulsifier, stabilizer and thickener, encapsulating agent, osmoregulation, and cryoprotectorin number of pharmaceutical and chemical industries such as food, cosmetics. It is used as plasma substitute, drug activity prolongator, radio-protector, and antitumor (Kang et al., 2003).

Searching for bioactive levan with unique feature is the target for many researchers. Previously, terrestrial and marine environments were reported as good sources for bacterial levan (Abdel-Fattah, Gamal-Eldeen, Helmy, & Esawy, 2012; Han & Watson, 1992). Two years ago, levan from honey microbial isolates attracted extensive interest due to their numerous biological activities (Esawy, Awad, et al., 2012). Honey dormant spores isolates characterized by their potentialities in the levansucrase production (Esawy, Ahmed, et al., 2012). Evaluation of *Bacillus subtilis* honey isolates as new source of levans yield was reported. These levans characterized by new properties such as antiviral and fibrinolytic activities (Esawy, Ahmed, et al., 2012; Salama, 2012). However, 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 through its scaling up. Previous studies on levan production

* Corresponding author. Tel.: +20 2 33335479; fax: +20 2 3370597.
** Corresponding author at: University of Natural Resources and Applied Life Sciences, Department of Chemistry, UFT-Haus Universitäts- und For schungszentrum Tulln (UFT), Konrad Lorenz Straße 24, 3430 Tulln an der Donau, Austria. Tel.: +20 2 33335479; fax: +20 2 3370597.
E-mail addresses: mona_esawy@hotmail.com, aya_esawy@hotmail.com (M.A. Esawy), hamer_1999@yahoo.com (H. Amer).

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were mostly carried out in shake-flask; however, it is obviously necessary to develop production process for this biopolymer in bioreactors. Characterization and fractionation of *Microbacterium laevaniformans* levan and cytotoxicity studies of their three levan fractions was reported (Kyung, Ho, BAE, Jaeho, & Lee, 2004). *Streptococcus salivarius*, levan was fractionated into 20 fractions series of varying molecular weights (Ehrlich et al., 1975). Fractionation gave us a good explanation of levan structure and bioactivity.

Levansucrase (2,β-fructan-6-β-fructosyltransferase, EC2.4.1.10) is an enzyme catalyzes levan synthesis from sucrose by transfructosylation reaction while releasing glucose in the medium (Jang et al., 2001). In addition to levan formation, levansucrase concomitantly produces fructooligosaccharides (FOS). FOS offer many applications in the food industry as a low-caloric sweetener and also as a factor promoting the growth of healthful microorganisms in the colon (Yun, 1996). Hence to achieve high production yields as well as to compete with synthetic petrochemical products in performance and cost, it is a prerequisite to develop a process for levansucrase production and its biopolymer in bioreactors.

Epithelial carcinogenesis is a multistep process in which an accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth, and, finally, carcinoma (Anne, Edward, & Waun, 2006). These affect different stages of carcinogenesis including initiation, promotion, and progression. Initiation involves direct DNA binding and damage by carcinogens and it is rapid and irreversible. Promotion, which involves epigenetic mechanisms, leads to pre-malignancy and is generally irreversible. Progression, which is due to genetic mechanisms, is the period between pre-malignancy and the cancer and is also generally irreversible. With rare exceptions, the stages of promotion and progression usually span decades after the initial carcinogenic exposure (Anne et al., 2006). The formation of reactive metabolites from procarcinogens is catalyzed by microsomal and nuclear monooxygenase enzyme systems consisting of NADPH-cytochrome P-450 (NADPH-CYP) reductase and isozymes of CYP. Once formed, reactive electrophiles may be detoxicated enzymatically either by conjugation with reduced glutathione, a reaction catalyzed by glutathione S-transferases (GST) (Cannady, Dyer, Christian, Sipes, & Hoyer, 2002).

In this study, one of the most our important goals was levan and levansucrase scaling up in stirred tank bioreactor. Accordingly, cell growth kinetics, levan, and levansucrase production during *Bacillus subtilis* M cultivations in shake flasks and In situ sterilizable pilot scale 16-l bioreactor under uncontrolled and controlled pH conditions was studied. This work also describes the isolation, fractionation and detailed NMR structural identification of the levan and examined the crude levan (levE) cancer chemo-preventive activity and their fractions, with special focus on their ability to prevent the initiation stage of carcinogenesis.

2. Materials and methods

2.1. Microorganism

The strain was previously isolated from a honey bee collecting nectar from desert flower (Libya). Honey is fresh non treated ripe honey (directly collected in beehives). The isolate was identified as *Bacillus subtilis* M based on molecular identification (Esawy et al., 2011). It was cultivated on Luria Bertani (LB) agar medium consisted of (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 (NalgenNunc Int., Rochester, NY, USA) and stored at −80 °C as master cell bank.

2.2. Cultivation conditions

Each experiment was started by two passage revival of one glycerol vial in liquid culture. First passage by using LB broth followed by production medium of the following composition (g/l): sucrose, 100, yeast extract, 2.0, K_2HPO_4, 3 and $MgSO_4{\cdot}7H_2O$, 0.2 (pH 7.0). Culture medium was inoculated with 250 μl of glycerol culture to 250 ml Erlenmeyer flask of 50 ml working volume. The inoculated flasks were incubated on a rotary shaker (Innova 4080, New Brunswick scientific Co., NJ, USA) at 200 rpm and 30 °C for 24 h. Cells were used thereafter to inoculate either 250 ml Erlenmeyer flasks or stirred tank bioreactor with 5% (v/v) inoculum concentration and inoculum density of 1 OD 600 nm.

2.3. Bioreactor cultivations

Cultivations in stirred tank bioreactor were conducted under the same cultivation conditions as in shake flasks in inoculum size terms, temperature and pH. The bioreactor used in this study was 16-l stirred tank bioreactor (Bio Engineering, Wald, Switzerland) with working volume of 8-l. The stirrer was equipped with two 6-blade Rushton turbine impellers (di (impeller diameter) = 85 mm, dt (tank diameter) = 214 mm, di/dt = 0.397). The agitation speed was adjusted to 100 rpm and kept constant during cultivation time. Aeration was performed using filtered sterile air and supplied continuously to the bioreactor with rate of 0.5 v v^{-1} min^{-1}. Foam was suppressed by silicon antifoam grade addition. pH and DO were determined throughout cultivation process using liquid filled pH electrode and DO polarographic electrodes respectively. In 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).

2.4. Levansucrase assay

Levansucrase assays were performed according to the method of Yanase et al. (1991) with some modifications (Abdel-Fattah, Mahmoud, & Esawy, 2005). Culture filtrate, 0.5 ml, was incubated with 1 ml 20% sucrose 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.One unit of enzyme activity was defined as the amount of enzyme that produced decreasing sugars equivalent to 1 mol glucose/min.

2.5. Polymer production and purification

The levan producing organisms were cultivated as described above. After the early stage of stationary phase, the culture was centrifuged at 5000 × g to remove cells isolate. The culture filtrate was dialized against deionized water for 48 h with dialysis membrane (M_r Cut off 10^4–12×10^3, diameter 60 mm) to remove the unfermented sucrose, and any fermentation products with low molecular weight. The dializate was frozen with liquid nitrogen and freeze dried to afford LevE.

2.6. Levan identification

2.6.1. Chromatographic analysis

Acid hydrolysis was done using 0.1 N HCl in boiling water bath for 1 h. Hydrolysis product was analyzed by descending paper-chromatography using Whatman No.1 and solvent system n-butanol:acetone:water (4:5:1, v/v/v) (Tanaka, OiLizuka, & Yamamoto, 1978) and sprayed with aniline phthalate (Block, Vurrum, & Zweig, 1955).

2.6.2. Molecular weight determination

Different LevE concentrations 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 control. Thus, specific viscosity/C (gsp) was estimated.

2.6.3. Total carbohydrates determination

After suitable dilution, 1 ml 5% phenol solution was added to 1 ml of the resulted diluted solution and (5 ml) H_2SO_4 (conc) were added rapidly to mixture, shake and set aside for 10 min at room temperature, then at 20-30 °C for 20 min Thereafter, the color density was measured at 490 nm Dubois, Gilles, Hamilton, Rebers, and Smith (1956).

2.6.4. ATR-FTIR spectroscopy

ATR-FTIR spectra from three samples (32 scans per sample, spectral resolution, 4 cm^{-1}, wave number range, 4000–700 cm^{-1} using a single reflection attenuated total reflectance (ATR) device (MIRacle, Pike Technologies, www.piketech.com) and a DLATGS detector) were recorded with a Bruker FT-IR spectrometer.

All samples used for infrared measurements were stored in a drying oven for three days at 50 °C and in a desiccator overnight over silica gel before measurement.

2.6.5. ^1H and ^{13}C NMR spectroscopy

NMR spectra were recorded at 27 °C unless otherwise stated with Bruker DPX 300 spectrometer (^1H at 300.13 MHz, ^{13}C at 75.47 MHz) employing standard Bruker NMR software. ^1H spectra in D_2O were referenced to DSS (4, 4-dimethyl-4-silapentane-1-sulfonic acid) in D_2O as external standard. ^{13}C NMR spectra were referenced to 1, 4 dioxane in D_2O as external standard. Coupling constants are reported in Hz and chemical shifts (δ) in ppm.

2.6.6. LevE fractionation

Freeze-dried dialysate was dissolved in water at a concentration of 10% (w/v) by stirring for 1 h. Isopropanol (99%) was slowly added under continuous stirring to obtain a final concentration of 66.5% at room temperature. Mixture kept for 2 h at 4 °C. The precipitated material was recovered by centrifugation (5000 × *g*, 30 min), The solvent was removed by rotary evaporation and the resulting precipitate re-dissolved in 50 ml deionized water, frozen with liquid nitrogen and freeze dried. The obtained fraction is referred to as F1. The supernatant was further saturated with isopropanol and the above procedure repeated (75% and 85% isopropanol) to afford F2, F3.

2.7. Cell culture

Murine hepatoma Hepa1c1c7 cells were purchased from the American Type Culture (CRL-2026). Cells were routinely cultured in Dulbeco's Modified Eagle's Medium (DMEM). Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 U/ml penicillin G sodium, 100 U/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells were maintained in humidified air containing 5% CO_2 at 37 °C. The monolayer cells were harvested using trypsin/EDTA. All experiments were repeated four times, unless mentioned, and the data were represented as (Mean ± S.E.). The levE and fractions were dissolved in DMSO (99.9%) and diluted 1000 fold in the assays. Results were compared with DMSO-treated cells. All cell culture material was obtained from Cambrex, BioScience (Copenhagen, Denmark).

2.8. Carcinogen metabolizing enzymes evaluation

2.8.1. Cytochrome P450 1A

Cytochrome P450 1A (Cyp1A) activity was determined by the dealkylation rate of 3-cyano-7-ethoxycoumarin (CEC) to fluorescent 3-cyano-7-hydroxycoumarin based on Crespi, Miller, and Penman (1997) and modified by Gerhäuser et al. (2003). Homogenates from cultured Hepa 1c1c7 cells induced with β-naphthoflavone were used as a Cyp1A source. A final concentration of 1.0 μg/ml of the LevE and fractions was used. Cyp1A activity inhibition was calculated in comparison with initial fluorescence of complete reaction mixture with cell homogenate and buffer instead of the samples.

2.8.2. Glutatione-S-transferase

Hepa 1c1c7 cells (1×10^6) were incubated with (10.0 μg/ml) of tested samples for 48 h. GSTs activity was measured in the cell lysate according to Habig, Pabst, and Jakoby (1974), based on GSTs-catalyzed reaction between GSH and 1-chloro 2,4-dinitrobenzene that acts as an electrophilic substrate for GSTs. In kinetic analysis, absorbance was assessed at 340 nm GSTs were normalized to the protein content as measured by bicinchoninic acid assay (Smith et al., 1985).

2.9. Oxygen radical absorbance capacity (ORAC)

The radical absorbance capacity of levE and fractions was tested against peroxyl ($ROO^•$) and hydroxyl ($OH^•$) by a kinetic ORAC assay (Cao & Prior, 1999) as modified by Gamal-Eldeen, Kawashty, Ibrahim, Shabana, and El-Negoumy (2004), by using β-Phycoerythrin (β-PE) as a radical-sensitive fluorescent indicator protein, 2,2,-azobis-(2-amidinopropane) dihydrochloride as an $ROO^•$ initiator and H_2O_2–$CuSO_4$ as $OH^•$ generator. β-PE fluorescence decay was measured using a microplate fluorescence reader at excitation 540 nm 205 and emission 565 nm. One ORAC unit equals the net protection of β-PE produced by 1.0 μM Trolox. Different concentrations of each tested sample were used (1.0, 2.0, and 4.0 μg/ml).

2.10. Splenocytes culture

Mouse splenocytes were used throughout the following in vitro experiments. Cells were routinely cultured in RPMI 1640 Media with 2 mM L-glutamine, was supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained in humidified air containing 5% CO_2 at 37 °C. Tested samples were applied to cultured mouse splenocytes at final concentration of 100 μg/ml. Cultured cells were treated with paclitaxil (TAX; 10 μg/ml). Cultured cells were treated with tested samples alone and in combination with TAX to evaluate tested materials bioactivity. Splenocytes were harvested using trypsin/EDTA and their viability was tested using trypan blue exclusion examination under a light microscope.

2.11. Micronucleus test

For measuring micronucleus (MN) test (Natarajan & Darroudi, 1991), splenocytes was seeded and incubated at (5.0×10^5 cells/ml, 37 °C, 48 h). Cytokinesis blocking agent, cytochalasin B (5 μg/ml) was added in fresh medium for 28 h before collecting cells. After incubation, splenocytes were re-incubated with tested samples for 24 h. Cells were trypsinized and soaked in cold hypotonic solution (KCl 5.6 g/l) for 20 min before fixed with methanol/glacial acetic acid (3:1) solution on glass slides. After air-drying of the slides, cells were stained with 2% Giemsa solution. All slides were scored,

and cells number with MN was recorded based on observation of 1000 binucleated cells for each concentration treatment.

2.12. Apoptosis and necrosis staining

Apoptosis and necrosis was investigated in treated and -untreated splenocytes using acridine orange/ethidium bromide staining (Gamal-Eldeen et al., 2004). A mixture of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml) was prepared in PBS. Stain cell uptake was monitored under fluorescence microscope where the apoptotic, necrotic, and viable cells were counted. Apoptotic cells exhibited membrane blebbing. Early and late apoptotic cells had yellow and orange chromatin respectively with nuclei that were highly condensed and fragmented. Necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, non biased manner.

2.13. DNA fragmentation assay

DNA fragmentation was essentially induced in vitro in mouse splenocytes by incubation with TAX (10 μg/ml) for 24 h in the presence or absence of the tested samples (100 μg/ml). Fragmentation was assayed as reported previously (Burton, 1956). Re-suspended in 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer), and incubated with an additional volume of lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 48 °C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13,000 × *g*. Pellets were re-suspended in TE-buffer and samples were precipitated by 10% trichloroacetic acid at 48 °C. Samples were pelleted at 4000 rpm for 10 min, mixed with 5% trichloroacetic acid, and then boiled for 15 min DNA content quantified using diphenylamine reagent (Messmer, Reed, & Brüne, 1995). Fragmented DNA percentage was calculated by the formula:

$$(\%)\,\text{DNA fragmentation} = \frac{\text{O.D. of supernatant}}{\text{O.D. of supernatant} + \text{O.D. of pellet}} \times 100$$

2.14. Statistical analysis

Statistical significance of treated and untreated cells was analyzed by using *t*-test; $p < 0.05$ indicated statistical significance.

3. Results

3.1. Cell growth, kinetics, levan, and levansucrase production

Bacillus subtilis M was cultivated in shake flask to study growth, levan and levansucrase production in small scale batch culture. Fig. 1 demonstrated growth profile and different metabolites production. As shown, cells grew exponentially without lag phase with 0.13 g/l/h growth rate and reached 0.9 g/l maximal cell mass after 9 h and kept more or less constant for the rest of cultivation time. For levan production, the biopolymer produced in culture in very low rate during early 3 h followed by high production rate (1.7 g/l/h) reaching about 18.6 g/l after 15 h and kept in stable titer in culture medium during the cultivation time. The levansucrease production profile in Fig. 1 showed short 2 h lag phase followed by high enzyme accumulation in culture medium (4.17 U/ml/h) rate. During the first 8 h pH medium dropped from 7 to 5 and increased gradually thereafter reaching 5.5 after 12 h and kept low acidic range during the cultivation.

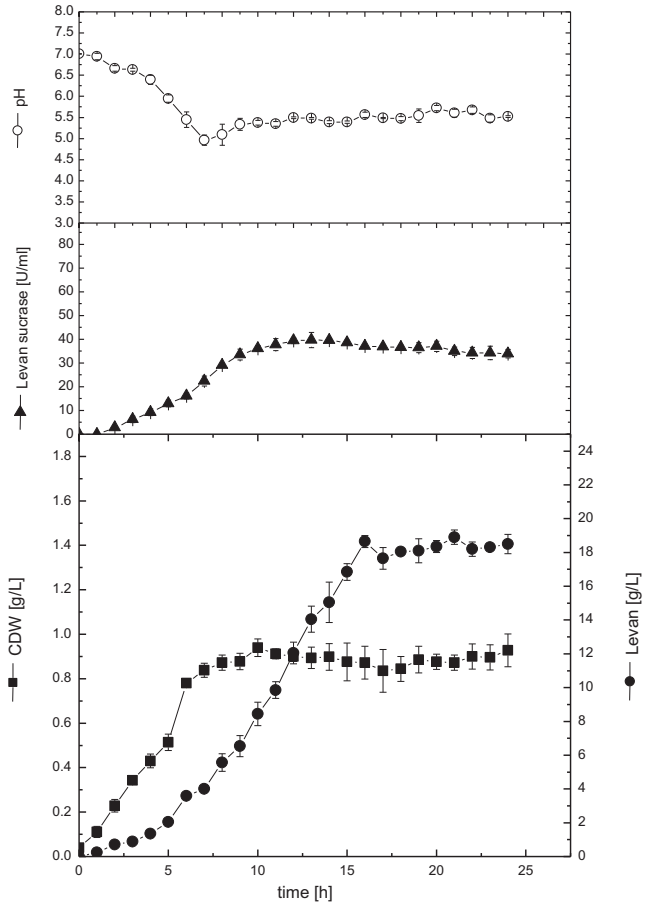

Fig. 1. Kinetics of cell growth, levan, levansucrase production during cultivation of *Bacillus subtilis M*-in shake flask.

All conditions were same as in shake flask experiment. Two parallel experiments were performed in stirred tank bioreactors of 8-l working volume for each and run under controlled (pH 7.0) and uncontrolled pH. Batch cultivation results without pH control are shown in Fig. 2. Cells grew exponentially without significant lag phase and reached 1.6 g/l after 10 h Cell and specific, growth rate were, 0.16 g/l/h and 0.2 g h^{-1}, respectively. Then, cells entered stationary and kept more or less constant for cultivation time rest. In parallel to cell growth, levansurcase produced in culture with 6.3 U/ml/h rate reaching 51.2 U/ml maximal values after 10 h. The enzyme concentration decreased gradually and reached about 30 U/ml after 25 h. After 2 h incubation levan concentration increased significantly in culture with 2.8 g/l/rate and reached 36.8 g/l maximal concentration after 15 h. During the active growth phase, the pH decreased gradually reaching about 4.3 after 5 h and kept more or less constant thereafter. Dissolved oxygen (DO) was decreased in culture as cell growth function, biopolymer production and reached about 30% saturation at cultivation time end.

In parallel, cultivation was conducted in other bioreactor under controlled pH. In this batch culture, pH was kept constant at 7 using computerized pH control system. In Fig. 3 cells grew exponentially (0.19 g/l/h growth rate) and reached 2.34 g/l after 11 h. During the active growth phase, levansurcase was produced with 9.1 U/ml/h rate reaching 74.1 U/ml after 9 h and kept more or less constant for the rest of cultivation time. In parallel to cell growth and enzyme production, levan was also produced in culture with constant rate during the active growth rate reaching 55 g/l after 11 h and increased with very low rate 6.3 g/l/h reaching 59.4 g/l maximal value after 24 h. During the active growth rate, the DO decreased significantly in culture and reached about 20% saturation as cells

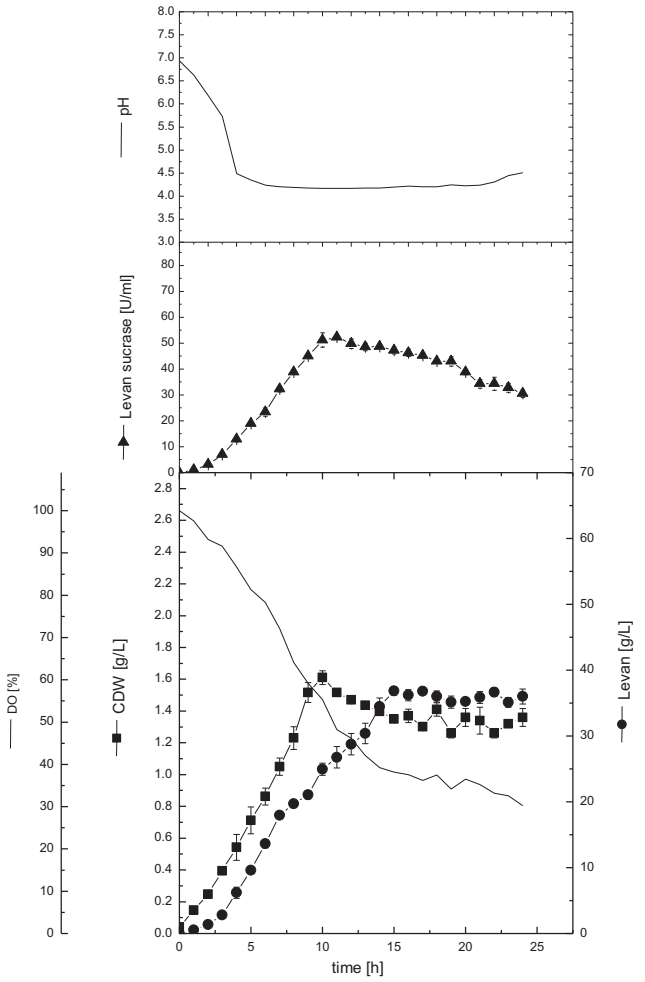

Fig. 2. Kinetics of cell growth, levan, levansucrase production during cultivation of *Bacillus subtilis M* in 16-l stirred tank bioreactor under uncontrolled pH condition.

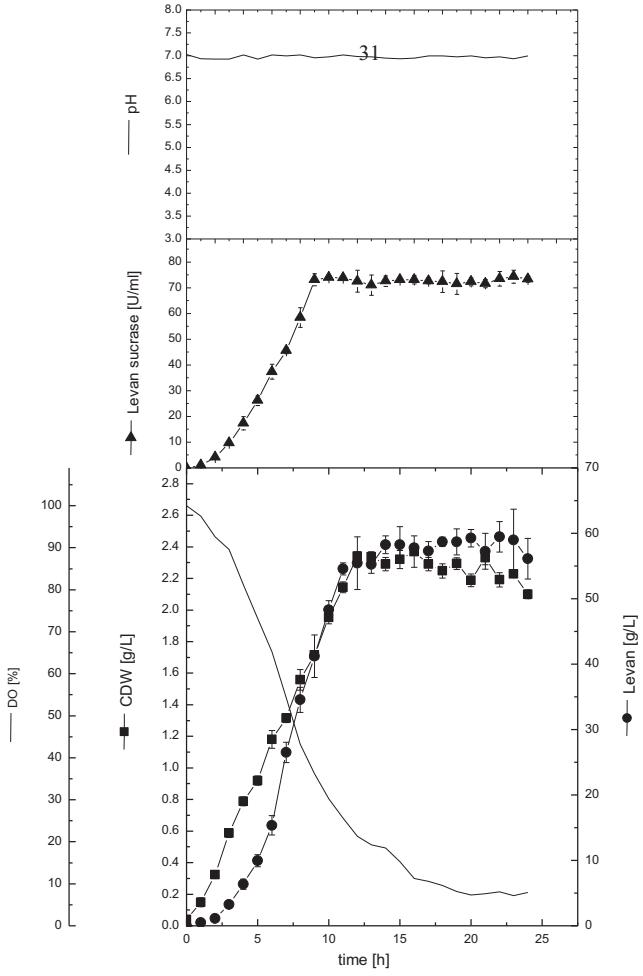

Fig. 3. Kinetics of cell growth, levan, levansucrase production during cultivation of *Bacillus subtilis M* in 16-l stirred tank bioreactor under controlled pH condition (pH 7).

entered the stationary phase. The DO decreased gradually thereafter as a function of biopolymer production in culture and reached only 8% saturation at the cultivation time end (24 h).

3.2. Chemical characterization of dialyzed (levE) and their fractions

LevE was fractionated into 3 fractions F1, F2, and F3. The molecular weight and total carbohydrate were 21.8, 13.118, and 9.53 kDa and 90.4, 94.5 and 78.8% respectively. The paper chromatography revealed that the main structure of F1, F2 was mainly fructose, and F3 was fructose and glucose in (3:1) ratio.

Spectral pattern of ^{1}H NMR spectrum (Fig. 4A) of levE showed skeletal protons in the region (δ 4.30–3.40) seven signals due to β-fructofuranosyl units, which were assigned by COSY and HSQC spectra. Because the detailed analysis of Lev proton spectra was not straightforward their 2D hetero-correlated HSQC spectra (Fig. 4B) were examined. ^{13}C NMR spectrum (Fig. 4C) showed main six signals at δ 61.22, 105.00, 77.11, 76.00, 81.09 and 64.19 were observed from the carbon C1, C2, C3, C4, C5 and C6, respectively, of fructosyl residues of Lev. Characteristic levan type cross peaks were well separated in the fructan-like polymer spectrum of the (Lev). H5/C5 at δ 3.96/81.09, and H6a, H6b/C6 at δ 3.90, 3.56/64.19 were used as levan type diagnostic cross peaks. The keto-anomeric signal C2 due to β-Fru*f* appeared at δ 105.00 ppm, C1 and C6 signals were detected at δ 60.73 and 64.18 ppm, respectively. 2,6-Linkages between β-Fruf units were confirmed by C2/H6 cross peaks δ 64.25 of β-Fru*f* in the HMBC spectrum (not shown) and confirmed the levan structure. The absence of the H1 signal due to α-glucose avoided a polymer molecular mass estimation from signal intensities in the 1H NMR spectrum. The appearance of four anomeric fructosyl signals (δ 104.90, 104.74, 104.59 and 104.31) in low intensities in Levan polymer indicated that high molecular weight with branched structure with 2,6 and 2,1-Linkages. The FTIR spectrum of the fructan-like polysaccharides exhibits some characteristic peaks, the most intensive absorption band of carbohydrates in IR-spectra are: strong complex absorption at 1080 cm^{-1}, and 1170 cm^{-1}, 1030 cm^{-1} (valent stretching vibrations of COC groups and vibrational modes in the composition of cyclic structures). Bands at 960 and 830 cm^{-1} are not so intensive, but useful for the conformational studies of carbohydrates. To identify the carbohydrate in the fractions F-1, F-2 and F-3, it is necessary to select the characteristic band. The comparison of crystalline fructose and glucose ATR-FTIR spectra (Fig. 5A) allowed selecting as characteristic bands of fructose: 780, 975, 1050, 1080, 1150, 1330 cm^{-1} and glucose: 995, 1020, 1050, 1105, and 1145. In ATR-FTIR study of three fructan-like polymer fractions F-1 and F-2 (Fig. 5B) the absorption bands in 700–1700 cm^{-1} and 2200–3700 cm^{-1} is similar for all fractions. Although the spectra were collected between 700 and 4000 cm^{-1}, the main differences in F-3 spectra appeared in 700–1700 cm^{-1} region compared with IR spectra of fructose, F-1 and F-2 indicated the presence of other carbohydrates. F-3 spectra,

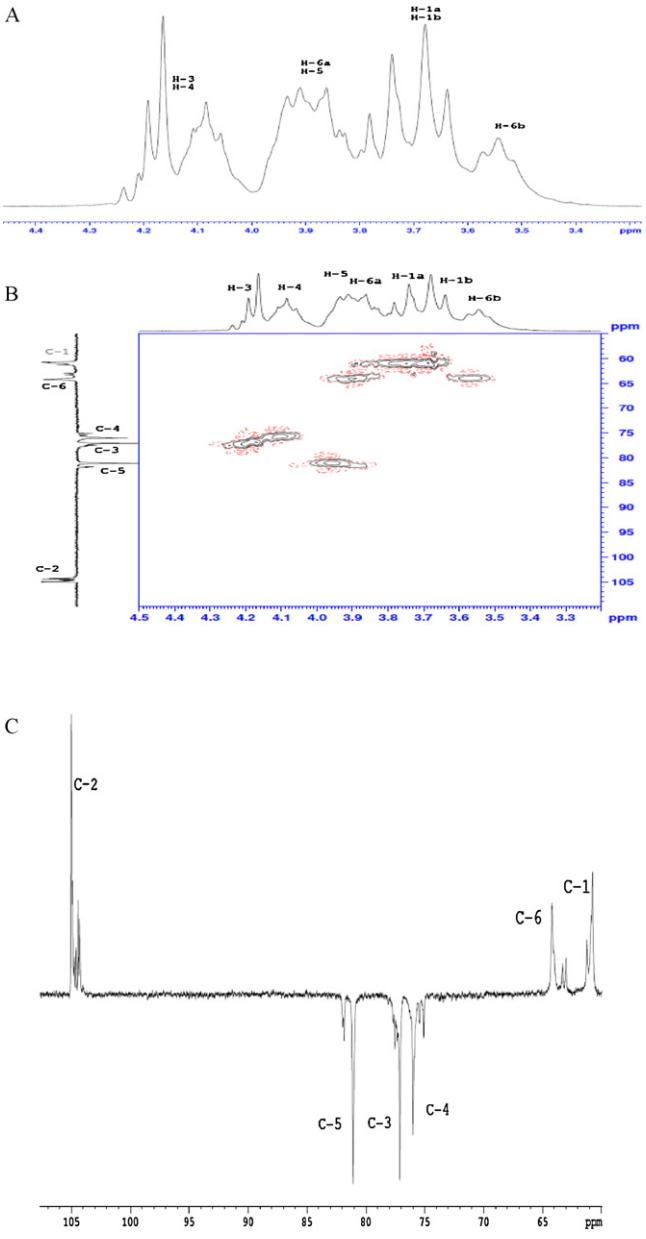

Fig. 4. ^{1}H- (A), HSQC (B) and ^{13}C-NMR (C) of levan product (Lev).

compared with glucose IR-absorption spectra, showed absorption bands similar to characteristic bands of glucose.

3.3. Evaluation of carcinogen metabolizing enzymes

The effect of crude levan (levE) levan and its three fractions on Cyp1A activity, as one of the enzymes involved in the activation of procarcinogens to ultimate carcinogens. Using Hepa 1c1c7-cells, our results revealed that total GSTs activity was significantly induced by the levE ($P < 0.05$) with an inhibition percentage of 54.4, and 71.2%, respectively (Fig. 6A), compared with the initial enzyme activity of β-naphthoflavone-stimulated cells. F1 and F2 possessed lower inhibitory effect on the Cyp1A activity.

The results revealed that total GSTs activity was significantly induced by the levE ($P < 0.05$) and fractions F3 ($P < 0.01$), while the rest of fractions led to insignificant change in the enzyme activity ($P > 0.05$) (Fig. 6B). Phase II enzymes generally conjugate activated xenobiotics to endogenous ligands.

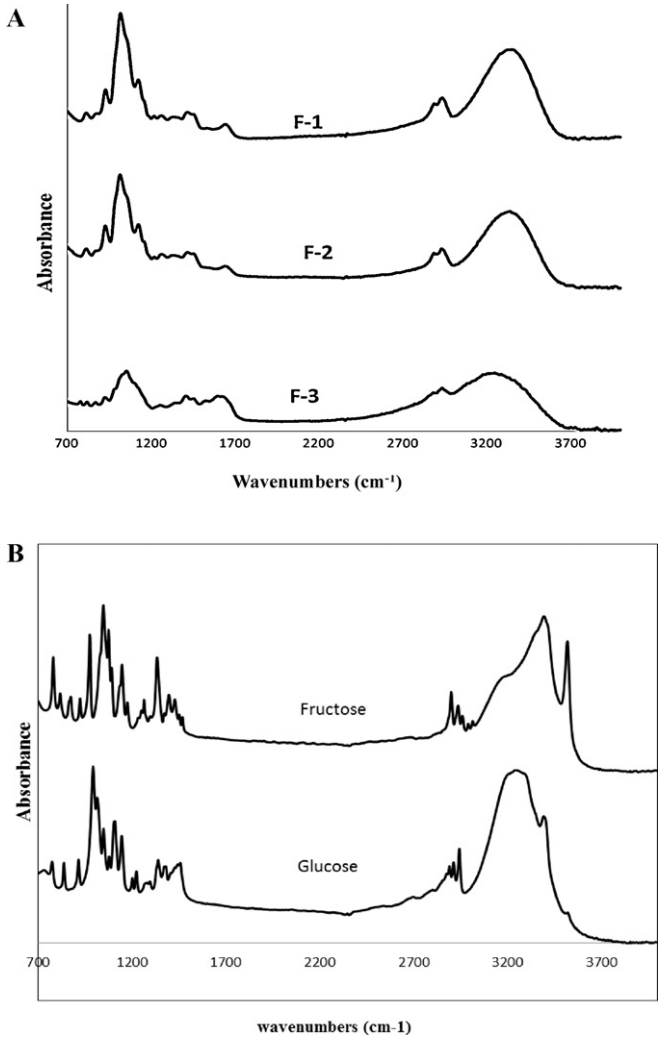

Fig. 5. (A) FTIR-ATR spectra of levan fractions (F-1, F-2 and F-3). (B) FTIR-ATR spectra of crystallized Fructose and Glucose.

3.4. Oxygen radical absorbance capacity (ORAC)

LevE and F3 were the strongest ROO• and OH• scavenger as indicated from their high ORAC units compared to the trolox capacity (Fig. 7A). F1 and F2 exhibited a relatively low scavenging affinity against ROO• and OH• as indicated from their low ORAC-OH• and ORAC-ROO• values (around one unit) (Fig. 7A).

3.5. Micronucleus test

Fig. 7B showed that nucleated damage induction was highly significant in TAX-treated splenocytes, since MN were significantly increased ($P < 0.001$). Splenocytes treatment with F1 resulted in a significant increase in MN containing cells ($P < 0.05$), compared to control cells. The post-treatment of TAX-treated splenocytes with levE and fractions remarkably suppressed TAX,

3.6. DNA fragmentation

Comparing the untreated with TAX-treated splenocytes indicated that TAX significantly induced the DNA fragmentation percentage ($P < 0.05$). Splenocytes treatment with TAX in combination with each fractions or levE resulted in a significant inhibition in the percentage of the TAX-induced DNA fragmentation as observed

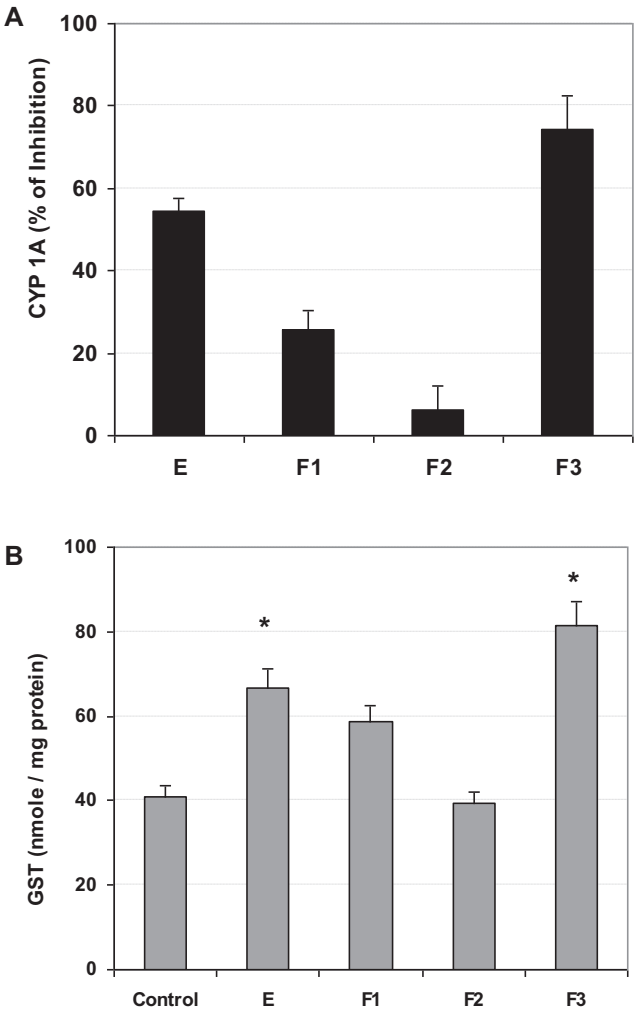

Fig. 6. (A) Anti-initiating activity through the modulation of the carcinogen metabolism: (A) the inhibitory effect of 1 μg/ml of each sample on CYP1A activity. Data was expressed as percentage of inhibition (Mean ± S.D.) in enzyme activity. (B) The effect of treatment with 10 μg/ml of each sample for 48 h on GSTs activity (bars) and on the non-enzymatic antioxidant GSH (circled line) in Hepa1c1c7 cells. Data was expressed as (Mean ± S.D.).

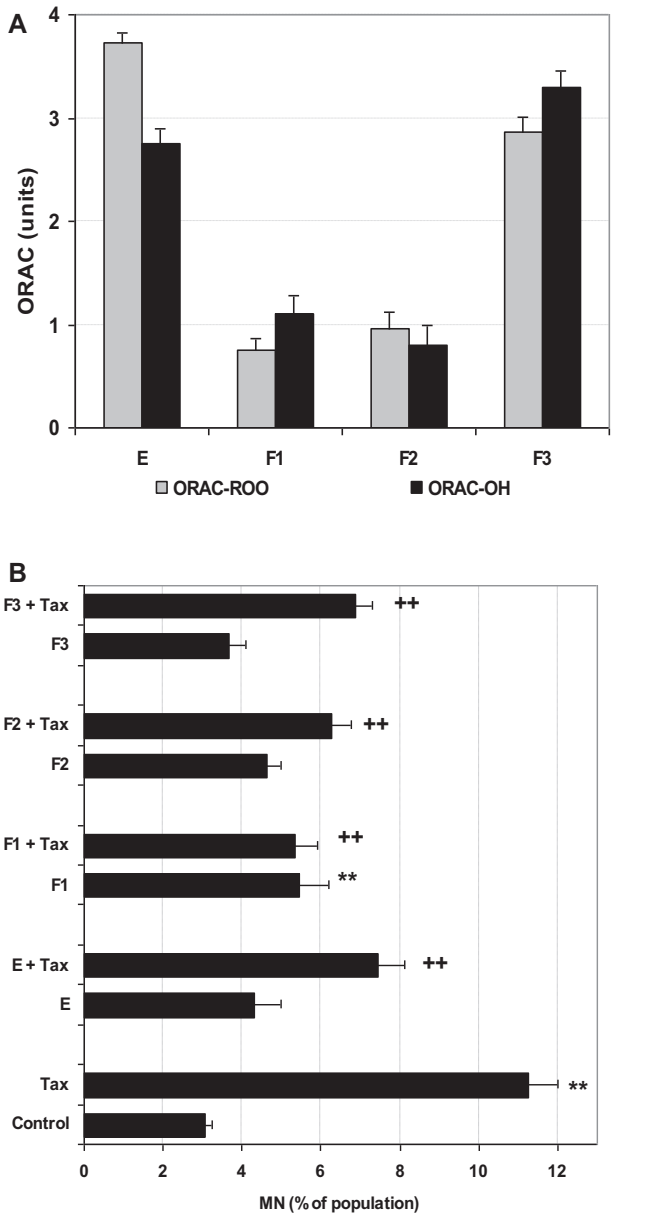

Fig. 7. (A) The antioxidant capacity of honeybee extract and its fractions, as assayed by ORAC assay, against OH• (black bars) and ROO• radicals (gray bars). (B) The effect of honeybee extract, its fractions, and paclitaxel (TAX) on the percentage of murine micronucleated splenocytes, as assayed by Giemsa stain. Data was expressed as (Mean ± S.D.).

when cells were treated with TAX in combination with F1 or F3, as shown in Fig. 8A.

3.7. Apoptosis and necrosis analysis

Fluorescence microscopic examination indicated that treatment with TAX triggered a significant apoptosis up to 45.6% ($P < 0.001$) in splenocyte population and that only F2 led to a similar triggered cell death by apoptosis up to 20.2% ($P < 0.05$) of the cells, while the treatment with other fractions and the levE resulted in a non-significant change in the apoptotic and necrotic populations ($P > 0.05$), as shown in Fig. 8B. Except F2, none of the tested samples triggered apoptosis nor necrosis in splenocytes.

4. Discussion

Honey contained a great variety of dominant spores and in consequence their dominant spores are expected to be new exopolysaccharide sources which could be isolated (Esawy, Ahmed, et al., 2012). This expectation comes from the honey constituents which is mainly fructose (about 38.5%) and glucose (about 31.0%) (Crosby Alfred, 2004). This investigation tries to continue in replying the question of whether honey collects bacteria that are beneficial for human or not (Esawy, Ahmed, et al., 2012; Esawy, Awad, et al., 2012). Previously honey isolate *Bacillus subtilis* M was identified as good levan producer characterized by antivirus and antioxidant activity (Esawy et al., 2011). Accordingly, the first aim in this study was *Bacillus subtilis* M levan scaling up to meet the industrial need with an efficient microbial method for increased levan production. Although many investigations on the levan production have been reported, all suffer from the disadvantages of low yield. Recently, strategies to improve the levan yield by microorganisms attracted greater attention (Melo, Pimentel, Lopes, & Calazans, 2007; Rhee et al., 2002). In this study, cultivation in bioreactor yielded higher biomass, biopolymer and enzyme production. This is directly related to better mixing and aeration in bioreactor compared to shake flask. *Bacillus* sp. belongs to highly aerobic microorganisms and need continuous supply of oxygen

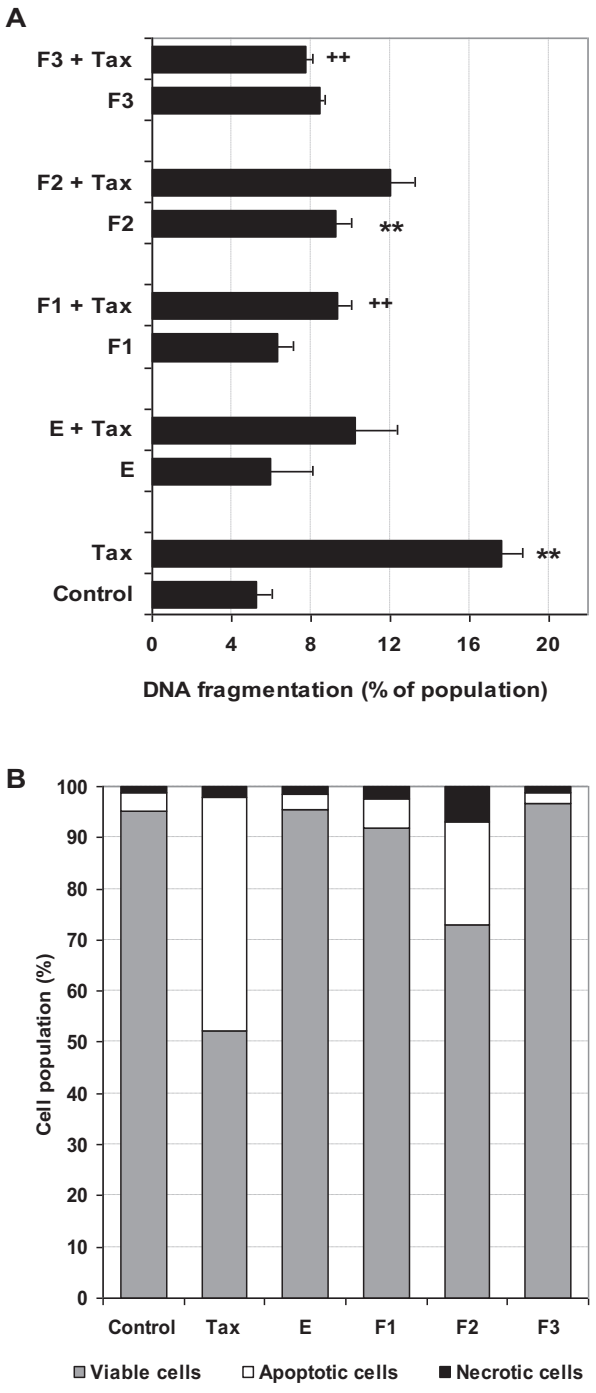

Fig. 8. (A) The effect of honeybee extract, its fractions, and paclitaxel (TAX) on the % of DNA fragmentation in splenocytes, compared with vehicle-treated cells. The amount of fragmented DNA was determined with the diphenylamine reaction (Mean ± S.D., $n = 4$). (B) Analysis of cell death type (apoptosis and necrosis) in splenocytes treated with honeybee extract, its fractions, and paclitaxel, as monitored by ethidium bromide/acridine orange staining (Mean ± S.D., $n = 4$).

for growth and bioactive metabolites synthesized through aerobic pathways. Cells cultivated in bioreactor culture under controlled pH condition yielded higher volumetric levan and levansucrase production compared to shake flask culture. Similar result was reported by Senthilkumar and Gunasegaram (2005), where the hydrolyzing activity and levan forming activity were both active at near neutral pH. The second part was levan fractionation and characterization depends on paper chromatography, ^{1}H NMR and ^{13}C NMR. All the previous tests confirmed each other and indicated that the 3 fractions were mainly fructose. Also, comparison of levan fractions and fructose spectra proved that fructose was the dominating component. Spectral data was in agreement with (Gurbe, Bekers, Upite, & Kaminska, 2002). Also, the spectral pattern was in strong accordance with (Matulová, Husárová, Capek, Sancelme, & Delort, 2011). Shih, Shieh, Yu, and Hsieh (2005) reported that ^{13}C NMR spectrum for levan produced by *B. subtilis Natto* (shows six main resonances at 60.1, 63.6, 75.4, 76.5, 80.5, and 104.4 ppm, which are almost identical with peak positions of our results, indicating that polysaccharide produced by *B. subtilis* M was levan type with linkage of β-(2, 6)-fructofuranoside. All fractions had low different molecular weights. Shih et al. (2005) reported in two levan fractions with high and low molecular weight F3 had glucose and reported low carbohydrate content compared to other fractions. The previous results indicated that F3 structure is widely difference comparing to F1 and F2.

F3 and levE were promising inhibitors of cancer chemopreventive activity and inducers of GST activity. Most chemical carcinogens require metabolic activation by Phase I enzyme in order to induce a biological response. Induction of Phase II drug-metabolizing enzymes such as GST is considered a major mechanism for protection against chemical stress and initiation of carcinogenesis (Bertram, 2000). Reactive metabolites production is largely dependent on primary metabolism by the cytochrome P450 enzymes. A key determinant of the cellular response to oxidative stress relates to the level and form of GSH, which helps in destruction of hydrogen peroxide, lipid peroxides and free radicals (Aggarwal & Shishodia, 2006). A major factor that affects GSH homeostasis is its utilization by conjugation, primarily via GSTs, which are responsible for the detoxification of a wide range of substrates including xenobiotics, and occupational and environmental carcinogens, and have evolved as a cellular protection system against their toxins and carcinogens (Rooseboom, Commandeur, & Vermeulen, 2004).

Conjugation and detoxification of active carcinogens is one of important mechanisms to halt tumor initiation step. Scavenging of the physiologically relevant ROS, including OH•, and ROO• represents an effective strategy in preventing tumor initiation and promotion. Results recorded that levE and F3 possessed the highest radical scavenging affinity to both of ROO• and OH• among the tested fractions. In oxidative stress and inflammation, excessive production of reactive oxygen and nitrogen species results in DNA damage and contributes to tumor initiation and promotion, which might ultimately lead to carcinogenesis. Cell protection depends on the availability and activity of cellular antioxidants, which maintain homeostatic control of ROS (Sun, Hail, & Lotan, 2004). An altered balance of ROS directly affects cellular proliferation, apoptosis, and senescence (Umar, Viner, & Haw, 2001). Although fractions F1 and F2 exhibited a relatively low scavenging affinity against ROO• and OH•, these affinities were close to that of the trolox capacity, as a known synthetic antioxidant. Reduced repair and/or oxidative stress by reactive oxygen will result in elevated DNA lesions, and accumulative mutations that lead to cancer. Consequently, scavenging of the physiologically relevant radicals, including OH•, and ROO• represents an effective strategy in preventing DNA damage.

Fractions and levE inhibited the paclitaxol-induced micronucleus formation in splenocytes. Only fractions 1 and 3 inhibited the induced-DNA fragmentation, this could be due to the observed radical scavenging activity against OH. Accumulation of such abnormalities in the genome is associated with cell transformation from a benign to a malignant phenotype. MNs assay provide a sensitive detection of freshly induced structural chromosomal damages in splenocytes (Fenech, 2000). They are small chromatin bodies that appear in the cytoplasm by the condensation of acentric chromosome fragments or by whole chromosomes and their frequencies have been considered to be a reliable index for detecting

chromosome breakages and loss (Martinez et al., 2005). MNs often induced by clastogenic substances or spindle-poison in dividing cells such as bone marrow (Fenech, 2000). None of the tested samples triggered apoptosis or necrosis in splenocytes, except fraction 2. Cell death is generally classified into two large categories: apoptosis, and necrosis. Necrosis is characterized by cell swelling, disruption and rapid disintegration of the cell membrane (Kalka, Ahmad, Criswell, Boothman, & Mukhtar, 2000). During, apoptosis the cells undergo nuclear and cytoplasmic shrinkage, the chromatin is condensed and fragmented, and the cells are finally broken into multiple membrane-surrounded bodies (apoptotic bodies) (Kalka et al., 2000). Results indicated that levE and its fraction 3 was active tumor anti-initiating and promising multi-potent blocking agent.

5. Conclusion

From all previous results, it was concluded that the levan production by *B. subtilis* M previously carried out in shake-flask process was successfully reproduced in a stirred tank bioreactor. Efficient levansucrase production and high levan yield were obtained under controlled conditions (pH = 7.0) after only 24 h. According to our knowledge, this is first systematic study of this strain in fermenter. The second part gave us clear explanation of the levan structure and helps us to discuss its cancer protective property clearly. The last part indicated that the levE and its fraction 3 were active tumor anti-initiating and promising multi-potent blocking agent.

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