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Levansucrase optimization using solid state fermentation and levan biological activities studies

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ABSTRACT

Bacillus subtilis NRC1aza produced levansucrase under solid state fermentation using starch as support. A sequential optimization strategy, based on statistical experimental designs is employed to enhance enzyme productivity. First, a 2-level Plackett–Burman design was applied for bioprocess parameters screen that significantly increase levansucrase production. Second optimization step was performed using fractional factorial design in order to optimize the amounts of highest positive variables that had significant effect on levansucrase productivity. Maximal enzyme productivity of 170 U/g ds was achieved in presence of glucose, yeast extract, and pH 8. *In vitro*, experiments confirmed that LevCR and LevQT had an antitumor activity against different animal and human cancer cell lines by demonstrating inhibitory effects on growth of Ehrlich ascites carcinoma cell line, human MCF-7 breast and liver HepG2 cancer cell lines, in particular LevQT was found to be efficacious compared to anticancer drug, cisplatin. Result focused in LevCR as strong fibrinolytic agent.

1. Introduction

Levansucrases, which are fructosyltransferases (E.C.2.4.1.9) belonging to glycoside hydrolases family 68, catalyzes formation of fructooligosaccharides (FOS) and synthesis of β-(2–6)-levan by transferring fructosyl group of non-activated sucrose into fructan chain (Dahech, Belghith, Belghith, & Mejdoub, 2012). FOS has been of increasing importance because of their favorable functionalities such as being low caloric, non-cariogenic and acting as growth factor for beneficial micro-organisms in intestinal flora (Padalino, Perez-Conesa, López-Nicolás, Frontela-Saseta, & Berruezo, 2012). Levan-type fructooligosaccharides has a very high molecular weight that can reach values of around 107 Da (Rhee et al., 2002). Levan offers industrial applications variety in cosmetics, foods and pharmaceuticals fields (Stivala & Bahary, 1978) as hypocholesterolemic agent (Moroti, Magri, Costa, Cavallini, & Sivieri, 2012). Recently, Esawy et al. (2011) reported in antivirus activity of levan isolated from honey *Bacillus subtilis*. However, antioxidant and antitumor activities of levan derivatives have been scarcely reported (Xue, Chen, Lu, & Jin, 2009; Zhang et al., 2009). On the other hand, natural polysaccharides derivatives studied recorded stronger antioxidant, or antitumor activities than their corresponding natural polysaccharides (Abdel-Fattah, Gamal-Eldeen, Helmy, & Esawy, 2012; Chen, Xu, Zhang, & Zeng, 2009; Liu, Luo, Ye, & Zeng, 2012). Thus, polysaccharides chemical modifications provide an opportunity to develop new agents with possible therapeutic uses.

Solid-state fermentation (SSF) produces product many-fold higher than that from submerged culture and has a relatively low energy requirement. Most of bacteria and fungi growing under SSF conditions capable of supplying global demand for various metabolites (Pandey, Szakacs, Soccol, Jose Rodriguez-Leon, & Soccol, 2001). SSF by low cost materials is considered to be best way especially in developing countries. Commercial starch as a support and substrate in SSF simulates a natural habitat for microorganisms. It is an inexpensive and abundantly available raw material. Enzyme production using starch as cost effective raw materials in SSF was reported by many authors (Chen, Chi, Chi, & Li, 2010; Soni, Kaur, & Gupta, 2003).

Optimization fermentation productivity has long been used in enhancing the yield of many bioprocesses. Optimization studies involving a one-factor-at-a-time approach is tedious and tends to overlook interacting factors effects but might lead to misinterpretations of results (Zhang et al., 1996). Optimization through factorial design and application of response surface methodology

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is a common practice in biotechnology for medium optimization components and process parameters (Abdel-Fattah, Saeed, Gohar, & El-Baz, 2005; Awad et al., 2011). It is collection of mathematical and statistical techniques for experiment design, model development, evaluation factors and optimum conditions of different biotechnological bioprocess. Statistical optimization not only allows quick screening of large experimental domain, but also reflects each components role.

Although there have been great advances in detection and treatment of cancer, it remains one of greatest medical challenges, with the incidence of some malignancies continuing to increase (Jemal et al., 2007). For many tumor types, established treatments such as cytotoxic chemotherapy and radiotherapy provide only transient therapeutic benefits despite severe side effects (Wilhelm et al., 2006). Therefore, needing for better treatments has stimulated research to develop new efficient chemotherapeutic agents for management of cancer.

Oxygen is essential molecule for all aerobic organisms, and plays predominant role in ATP generation, namely, oxidative phosphorylation. During this process, reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are produced as by-products (Fang, Seki, & Maeda, 2009), under physiological conditions; cells have a series of defense systems to counteract these reactive insults. Such defense systems include intracellular superoxide dismutase (SOD) that converts $O_2^{\bullet -}$ to H_2O_2, catalase and glutathione peroxidase that eliminate H_2O_2, in addition to free radical scavenging compounds like glutathione (Fang et al., 2009).

Tumor cells have higher ROS levels and are more frequently deficient in most crucial antioxidative enzymes than normal cells and are therefore more vulnerable to additional oxidative stress (Pelicano, Carney, & Huang, 2004). Accordingly, a unique antitumor strategy named "oxidative therapy" was developed by delivering excess oxidative stress or disrupting antioxidative defense system in cancer cells (Huang, Feng, Oldham, Keating, & Plunkett, 2000). Moreover, Abdala-Díaz, Chabrillón, Cabello-Pasini, López-Soler, and Figueroa (2010) reported that, release of NO by macrophages is also known to be involved in anti-tumor defense.

To best of our knowledge, SSF technique is yet to be explored for levansucrase production in SSF by using starch as substrate. Levansucrase production was carried out through a stepwise optimization strategy including, at first medium and environmental components elucidation that affect enzyme productivity significantly using Plackett–Burman design then optimization of most significant components by Fractional factorial design creating a mathematical model expressing relationship between optimized factors and levansucrase production. Levan was isolated and chemically modified. Crude (LevCR) and quaternized levan (LevQT) were identified by paper chromatography and ^{1}H NMR. Hence, anticancer effect of LevCR and LevQT investigated on different cell lines and to elucidate the mechanism whereby the prepared compounds exert their antitumor activities, free-radical-metabolizing enzymes as well as oxidative stress parameters levels in different cell lines were estimated. Moreover, *in vitro* experiment was done to evaluate (LevCR) and (LevQT) anticancer activity against Ehrlich ascites tumor cells as animal cancer model. Also, levan fibrinolytic activity was tested.

2. Materials and methods

2.1. Microorganism and maintenance

Bacterium used throughout this work, *Bacillus subtilus* NRC1aza was previously isolated and identified (Abdel-Fattah et al., 2012). Bacterial strain was routinely grown on nutrient agar medium at 30 °C and preserved at −80 °C in 50% (v/v) glycerol.

2.2. Cultivation conditions and crude enzyme extraction

Ten grams of each substrate (lupin, wheat bran, ground barely, semolina flour, arena, rice straw, starch, plain flour and yellow maize flour) were taken separately into 250 ml Erlenmeyer flasks and moistened with 10 ml medium consisted of (g/l): yeast extract, 2.5; sucrose, 80; $MgSO_4$, 0.2; and K_2HPO_4, 5.5. Medium components were dissolved in 1 l distilled water; pH was adjusted to 7.8. Flasks were autoclaved for 20 min at 121 °C and cooled to room temperature before inoculation. Sterilized solid substrate was inoculated with 2 ml inoculums (1.2×10^6 CFU/ml) of the overnight culture in nutrient broth medium. Culture flasks were then incubated at 37 °C for 24 h. At incubation time end, extracellular enzyme was prepared by 100 ml of 0.01 M sodium phosphate buffer addition (pH 7.0) to the medium followed by centrifugation at 5000 rpm for 20 min, the supernatant was used in enzyme assay.

2.3. Levansucrase assay

Levansucrase assay was performed according to Yanase et al. (1991) method. Decreasing amounts of sugars produced were measured by glucose oxidase kits. One unit of enzyme activity was defined as amount of enzyme that produced decreasing sugars equivalent to 1 μmol of glucose/min.

2.4. Precipitation of levan from B. subtilis NRC1aza culture

Levan was isolated from culture filtrate of *B. subtilis* NRC1aza through precipitation with (2:1, v/v) 96% ethanol: culture filtrate. The solution were mixed and left for 24 h at room temperature. Precipitated levan was separated from the culture filtrate by centrifugation at 5000 rpm for 10 min.

2.5. Levan identification and its modification

2.5.1. 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, Lizuka, & Yamamoto, 1978) and sprayed with aniline phthalate (Block, Vurrum, & Zweig, 1955).

2.5.2. Nuclear magnetic resonance (NMR)

NMR spectra were recorded at 27 °C unless otherwise stated with a Bruker DPX 300 Spectrometer (^{1}H at 300.13 MHz, ^{13}C at 75.47 MHz) employing standard Bruker NMR software. ^{1}H 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.

2.5.3. Preparation of quaternized levan

Quaternization of levan was carried out according to (Lim & Hudson, 2004) method with some modifications. Briefly, levan (0.5 g) was dissolved in 2.5 ml of distilled water, 1.5 g of 2,3-epoxypropyl triethyl ammonium chloride was added, the mixture well mixed and left on shaking water bath at 40 °C for 48 h. Subsequently, the solution was purified using dialysis technique for 72 h against distilled water (about 3 l) and the process was repeated 3–4 times till clear solution was obtained, then precipitation was carried out by adding acetone, the precipitate was lyophilized for 24–48 h to give quaternized levan

Table 1
Coded levels and real values for Plackett–Berman experiment.

Trial no.	Culture condition		Carbon sources		Nitrogen sources			Energy sources		Metal ions				Levansucrase activity (U/gds)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	
	pH	Moisture content	Sucrose	Glucose	Yeast extract	Peptone	$(NH_4)_2SO_4$	KNO_3	K_2HPO_4	KH_2PO_4	$MgSO_4$	$CuSO_4$	$FeSO_4$	$ZnSO_4$	$MnSO_4$	
1	−1(6)	+1(100)	+1(140)	+1(100)	−1(0.5)	+1(2)	−1(0.5)	−1(0.5)	+1(5)	+1(5)	+1(0.2)	−1(0)	+1(0.001)	−1(0)	+1(0.001)	5.8 ± 0.669
2	+1(8)	−1(50)	+1(140)	−1(60)	−1(0.5)	−1(0.5)	−1(0.5)	+1(2)	+1(5)	+1(5)	−1(0.02)	+1(0.001)	−1(0)	+1(0.001)	−1(0)	13.81 ± 3.701
3	+1(8)	+1(100)	−1(60)	+1(100)	−1(0.5)	−1(0.5)	+1(2)	−1(0.5)	+1(5)	−1(2)	+1(0.2)	−1(0)	+1(0.001)	+1(0.001)	+1(0.001)	20.92 ± 5.63
4	−1(6)	+1(100)	+1(140)	−1(60)	+1(2)	−1(0.5)	+1(2)	+1(2)	−1(2)	+1(5)	−1(0.02)	+1(0.001)	−1(0)	+1(0.001)	+1(0.001)	13.18 ± 1.378
5	+1(8)	−1(50)	−1(60)	−1(60)	+1(2)	+1(2)	+1(2)	+1(2)	+1(5)	−1(2)	+1(0.2)	−1(0)	+1(0.001)	−1(0)	−1(0)	14.25 ± 6.102
6+	−1(6)	+1(100)	−1(60)	−1(60)	+1(2)	+1(2)	−1(0.5)	−1(0.5)	−1(2)	+1(5)	−1(0.02)	+1(0.001)	+1(0.001)	+1(0.001)	+1(0.001)	10.00 ± 5.118
7	−1(6)	−1(50)	−1(60)	+1(100)	−1(0.5)	+1(2)	+1(2)	+1(2)	+1(5)	−1(2)	+1(0.2)	+1(0.001)	−1(0)	−1(0)	−1(0)	13.07 ± 2.165
8	−1(6)	−1(50)	+1(140)	+1(100)	+1(2)	−1(0.5)	−1(0.5)	−1(0.5)	−1(2)	+1(5)	+1(0.2)	−1(0)	+1(0.001)	+1(0.001)	−1(0)	10.88 ± 1.575
9	+1(8)	−1(50)	+1(140)	+1(100)	−1(0.5)	+1(2)	+1(2)	−1(0.5)	+1(5)	+1(5)	−1(0.02)	+1(0.001)	−1(0)	−1(0)	−1(0)	20.81 ± 0.512
10	+1(8)	+1(100)	+1(140)	−1(60)	+1(2)	−1(0.5)	−1(0.5)	−1(0.5)	+1(5)	−1(2)	+1(0.2)	−1(0)	−1(0)	+1(0.001)	+1(0.001)	25.18 ± 4.685
11	+1(8)	+1(100)	−1(60)	+1(100)	−1(0.5)	+1(2)	+1(2)	+1(2)	−1(2)	+1(5)	−1(0.02)	−1(0)	−1(0)	+1(0.001)	+1(0.001)	32.00 ± 2.677
12	−1(6)	−1(50)	+1(140)	−1(60)	+1(2)	−1(0.5)	+1(2)	+1(2)	+1(5)	−1(2)	−1(0.02)	−1(0)	+1(0.001)	−1(0)	−1(0)	18.37 ± 1.85
13	+1(8)	+1(100)	−1(60)	+1(100)	+1(2)	+1(2)	−1(0.5)	+1(2)	−1(2)	−1(2)	−1(0.02)	+1(0.001)	+1(0.001)	+1(0.001)	+1(0.001)	28.00 ± 2.047
14	−1(6)	−1(50)	+1(140)	−1(60)	−1(0.5)	+1(2)	+1(2)	−1(0.5)	−1(2)	−1(2)	+1(0.2)	+1(0.001)	+1(0.001)	−1(0)	−1(0)	6.00 ± 2.402
15	+1(8)	+1(100)	1(60)–	+1(100)	+1(2)	−1(0.5)	−1(0.5)	+1(2)	−1(2)	+1(5)	+1(0.2)	+1(0.001)	−1(0)	−1(0)	+1(0.001)	14.03 ± 1.457
16	−1(6)	−1(50)	1(60)–	−1(60)	−1(0.5)	−1(0.5)	−1(0.5)	−1(0.5)	−1(2)	−1(2)	−1(0.02)	−1(0)	−1(0)	−1(0)	−1(0)	3.00 ± 1.16

Real values (given in parentheses) are in g/l.

Table 2
Statistical analysis of Plackett–Burman design showing coefficient values, *t*- and *p*-values and percent of confidence level for each variable on levansucrase activity.

Variables	Coefficient	*t*-statistics	*p*-value	Confidence level (%)
Intercept	9.453			
pH	3.858	5.097087	0.0002	99
Moisture content (%)	2.729	1.087379	0.295	70
Sucrose	0.429	2.823786	0.0135	98
Glucose	−3.835	−1.04427	0.314	68
Yeast extract	0.805	2.96699	0.01	99
Peptone	−1.434	−0.84587	0.4118	58
$(NH_4)_2SO_4$	−3.022	−0.45485	0.656	34
KNO_3	−3.591	−1.40194	0.182	81
K_2HPO_4	−2.926	0.630097	0.5388	46
KH_2PO_4	0.037	2.919903	0.011	98
$MgSO_4$	−1.652	−0.1318	0.897	10
$CuSO_4$	1.873	0.669903	0.5137	48
$FeSO_4$	1.403	1.796117	0.0941	90
$ZnSO_4$	2.674	0.598058	0.559	44
$MnSO_4$	v2.425	−2.06675	0.057	94

2.6. Experimental designs

2.6.1. Plackett–Burman design

Plackett–Burman experimental design was used to evaluate the relative importance of various nutrients for levansucrase production by *B. subtilis* NRC1aza in SSF. Fifteen components were selected for the study, each variable represented at two levels, high concentration (+1) and low concentration (−1) in 16 trials as shown in Table 1 (Plackett & Burman, 1946). Tested factors included pH, moisture content, glucose, sucrose, peptone, yeast extract, $(NH_4)_2SO_4$, KNO_3, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, $CuSO_4$, $FeSO_4$, $ZnSO_4$ and $MnSO_4$. Each row represented a trial run and each column represented an independent variable.

Plackett–Burman experimental design is based on first order model:

$$Y = B_0 \sum B_i x_i \tag{1}$$

where Y is the response (levansucrase production), B_0 is model intercept and B_i was variables estimates. Effect of each variable was determined by following equation:

$$E(X_i) = \frac{2\left(\sum M_I^+ - M_I^-\right)}{N} \tag{2}$$

where $E(X_i)$ is tested variable effect. M_i^+ and M_i^- represent levansucrase production from trials where variable (X_i) measured was present at high and low concentrations, respectively and N is the number of trials in Eq. (2).

Standard error (SE) of the concentration effect was square root of the variance of an effect, and the significance level (p-value) of each concentration effect was determined using Student's t-test $t(X_i)$.

$$t(X_i) = \frac{E(X_i)}{\mathrm{SE}} \tag{3}$$

where $E(X_i)$ is variable X_i effect.

2.6.2. Fractional factorial design (FFD)

The most promising variables achieved the highest levansucrase productivity by Plackett–Burman experimental design (pH, glucose, yeast extract and KH_2PO_4) were chosen for response surface methodology of FFD (Rosa, Soria, Vélez, & Galvagno, 2010). Variables were studied at three different levels. Experimental design used for this study and the statistical analysis were shown in Tables 2 and 3. All experiments were done in triplicate and levansucrase production average obtained was taken as dependent variable or response (Y). The second-order polynomial coefficients

Table 3
Fractional factorial design (FFD) and response levansucrase activity by *Bacillus subtilus* NRC1aza.

Trial	Factor levels									
	pH (X_1)		Sucrose (X_2)		Yeast extract (X_3)		KH_2PO_4 (X_4)		Levansucrase activity (U/g ds)	
	Coded	Actual g/l	Coded	Actual g/l	Coded	Actual g/l	Coded	Actual g/l	Experimental	Predicted
1	+1	8	+1	100	−1	0.5	−1	1.0	89.38	88.21
2	+1	8	+1	100	+1	2.0	−1	1.0	30.0	36.7
3	−1	6	+1	100	−1	0.5	−1	1.0	40.0	40.48
4	0	7	0	80	0	1.0	0	2.0	114.74	99.170
5	−1	6	+1	100	+1	2.0	−1	1.0	68.58	68.90
6	+1	8	+1	100	+1	2.0	+1	3.0	15.0	19.70
7	−1	6	−1	60	+1	2.0	+1	3.0	108.22	103.36
8	+1	8	−1	60	+1	2.0	−1	1.0	20.0	20.15
9	+1	8	−1	60	−1	0.5	−1	1.0	87.22	82.12
10	−1	6	+1	100	+1	2.0	+1	3.0	90.22	79.07
11	−1	6	−1	60	−1	0.5	−1	1.0	10.0	2.18
12	−1	6	−1	60	−1	0.5	+1	3.0	72.19	77.86
13	+1	8	−1	60	+1	2.0	+1	3.0	98.77	80.59
14	0	7	0	80	0	1.0	0	2.00	106.67	99.17
15	+1	8	−1	60	−1	0.5	+1	3.0	170.0	164.36
16	−1	6	−1	60	+1	2.0	−1	1.0	51.0	45.11
17	−1	6	+1	100	−1	0.5	+1	3.0	79.35	72.44
18	0	7	0	80	0	1.0	0	2.0	92.0	97.81
19	+1	8	+1	100	−1	0.5	+1	3.0	132.0	122.35

F-value = 7.58; $p > F = 0.006$; $R^2 = 0.923$; $R = 0.801$; adjusted $R^2 = 0.961$.

were calculated and analyzed using 'SPSS' software (Version16.0) Second degree polynomials, Eq. (4), which includes all interaction terms, β were used to calculate predicted response:

$$Y_{\text{activity}} = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (4)$$

where Y_{activity} represented levansucrase activity, β_0 is interception coefficient, β_i was the coefficient of the linear effect, β_{ii} were quadratic effect coefficients and β_{ij} were cross product coefficients, X_iX_j were independent variables which influence response variable Y. Model statistical analysis was performed to analysis variance evaluation (ANOVA). Model statistical significance was determined by Fisher's test value, and variance proportion explained by the model was given by multiple coefficient determination for each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated by using STATISTICA (0.6).

2.7. Determination of in vitro antitumor activity-cytotoxicity assay on Ehrlich ascites tumor cells

2.7.1. Ehrlich ascites cells and animals

Animal care and handling were done according to guidelines set by World Health Organization, Geneva, Switzerland, and according to approval from ethical committee for animals care at National Research Centre, Egypt. Female Swiss albino mice (8–10 weeks) weighting 22–25 g were used. Animals were housed in polycarbonate cages at 25 °C, 12 h day–night cycle and 45–65% humidity. Fed was done with balanced commercial diet and water.

Ehrlich ascites tumor cells, derived from a spontaneous murine mammary adenocarcinoma, were maintained in the ascites form by peritoneal transplantation of 2×10^6 cells resistant to Endoxan. Ascitic tumor cell counts were done in a Neubauer hemocytometer. Cell viability was checked by trypan blue dye exclusion method to be more than 99% viable (Amer, Helmy, & Taie, 2010; El-Merzabani, El-Aaser, Attia, El-Duweini, & Ghazal, 1979).

Different concentrations of LevCR and LevQT were dissolved in phosphate-buffered saline (PBS) at a final concentrations of (200, 400, 600, 800, 1000 μg/ml). Tumor cells were taken from peritoneal cavities of tumor-bearing mice and diluted with 0.1 M phosphate buffered pH 7 at final concentration 2.5×10^5 cells/μl. In a sterile test tube10 μl of tested samples was added to10 μl of tumor cell and 80 μl of PBS buffer. Tubes were incubated at 37 °C for 2 h under 5% CO_2. They were centrifuged at 1000 rpm for 5 min; remaining viable cells were washed twice with PBS buffer and then re-suspended to previous final volume in the same buffer. Trypan blue (10 μl) was added and mixed thoroughly for tested samples and controls. Control was cell preparations without addition of tested samples. Numbers of living cells were calculated using a hemocytometer slide. Survived cells appeared as unstained bodies while non-viable cells stained blue (El-Merzabani et al., 1979).

2.8. Cell lines and culturing

Anticancer activity screening for the tested compounds utilizing 3 different human tumor cell lines including breast cancer cell line MCF-7; human liver cancer cell line HepG2 and human lung cancer cell line A549 were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50×10^6 were grown in a 25 cm^2 flask in 5 ml of complete culture medium.

2.9. In vitro antiproliferative assay

The antiproliferative activity was measured *in vitro* using the Sulfo-Rhodamine-B stain (SRB) assay according to the previous reported standard procedure (Skehan et al., 1990). Cells were inoculated in 96-well microtiter plate (10^4 cells/well) for 24 h before treatment with the tested compounds to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the compounds under test (0, 20, 50, 100 and 200 μg/ml) were added to the cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37 °C and in atmosphere of 5% CO_2. After 48 h cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is

Table 4
Cytotoxicity (IC_{50}, μg/ml) of LevCR and LevQT against different human cell lines.

Compound	Cell line		
	MCF-7	Cell line HepG2	A549
Cisplatin	18.0	27.0	N.A.
LevCR	43.0	64.0	N.A.
LevQT	30.0	44.0	N.A.

N.A., no activity.

plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Table 4.

2.10. Biochemical assays

The cells in culture medium were treated with 20 μl of 1/10 of IC_{50} values of the compounds or the standard reference drug, cisplatin, then incubated for 24 h at 37 °C, in a humidified 5% CO_2 atmosphere (Ali, Mahmoud, & Rashad, 2011). The cells were harvested and homogenates in saline using a tight pestle homogenizer until complete cell disruption for further biochemical analysis. The supernatants obtained after centrifugation of cell homogenates was used for further investigations.

2.11. Antioxidant enzyme and oxidative stress assays

The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) were determined as described by Aebi (1984), Marklund and Marklund (1974), respectively. The levels of hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) were determined by the methods of Wolff (1994), Montgomery and Dymock (1961), Ellman (1959) respectively.

2.12. Estimation of nucleic acids and protein

Nucleic acids (DNA and RNA) and total protein were precipitated and measured in cell homogenates. Total DNA was extracted and assayed according to the method described by Ali, Frei, Straub, Breuer, and Wiessler (2002), total RNA was extracted and assayed according to the method adopted from the method provided by Hybaid/AGS (Germany), and total cellular protein was assayed by the method of Lowry, Rosebrough, Farr, and Randall (1951).

2.13. Evaluation of fibrinolytic activity

Fibrinolytic activity of LevCR and LevQT was performed as described in (Helmy, Amer, & EL-Shayeb, 2007) Sets of three-hard glass test tubes (31 mm × l00 mm) were cleaned by immersion overnight in chromic acid. To each tube 0.8 ml of 0.9% saline solution, 1 ml plasma and 0.2 ml of 2% calcium chloride solution were added. After mixing, the tubes were placed in water bath at 37 °C and when clotting was complete, 2000 μg of heamoclar or tested samples was added individually. After 30 minute of incubation at 37 °C. Lyses percentage of plasma clots was recorded by using measuring cylinder.

3. Results

3.1. Evaluation of different substrate for levansucrase production

The present study indicated that levansucrase production pattern varied with substrate type. Maximum enzyme production (36.00 ± 0.247 U/g ds) was observed with starch, followed by yellow maize flour (32.00 ± 0.129 U/g ds) and wheat bran (30.00 ± 0.707 U/g ds) while minimum production (10.59 ± 0.289 U/g ds) was noticed with semolina flour as substrate/support material (data not shown).

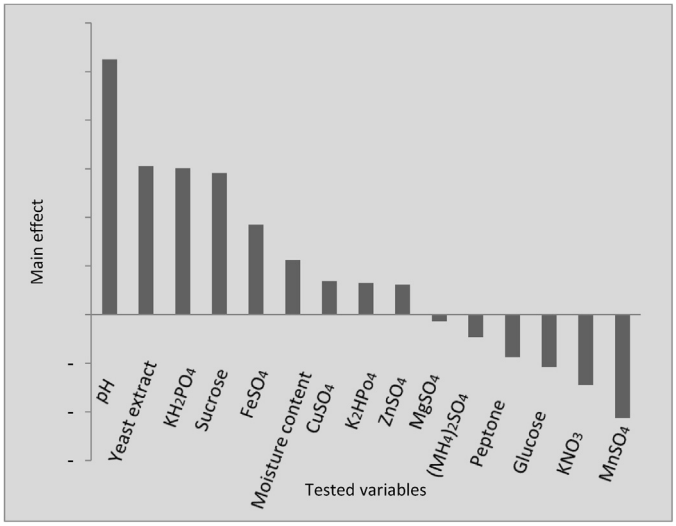

Fig. 1. Effect of culture conditions and medium composition on levansucrase (U/g ds) produced by *B. subtilis* NRC1aza.

3.2. Levansucrase production optimization by multi-factorial experiments

Sequential optimization approaches were applied in this study. First approach deals with screening for nutritional factors affecting growth of *B. subtilis* NRC1aza with respect to levansucrase production. Second approach is to optimize the factors that control the enzyme production process.

3.3. Evaluation of the factors affecting levansucrase productivity

In first approach, Plackett–Burman design was used. Fifteen different factors (variables) including culture conditions and medium constitution were chosen to perform this optimization process. Levansucrase activity averages for different trials were given in U/g ds and shown in Table 1. Main effect of each variable on levansucrase activity was estimated as difference between both measurements averages made at high (+1) and low levels (−1) of that factor. Data in Table 1 showed a wide variation from 3.00 ± 1.16 to 32.00 ± 2.677 U/g ds on levansucrase productivity. Data analysis of the Plackett–Burman experiments involved a first order model. The main effects of the examined factors on enzyme productivity and their ranking were calculated and presented graphically in Fig. 1.

Regression coefficients analysis of the tested variables showed that pH, yeast extract, KH_2PO_4, sucrose, $FeSO_4{\cdot}7H_2O$, moisture content $CuSO_4{\cdot}7H_2O$, K_2HPO_4, $ZnSO_4{\cdot}7H_2O$ showed positive effect on levansucrase productivity while, $MgSO_4{\cdot}7H_2O$, $(NH_4)_2SO_4$, peptone, glucose, KNO_3, $MnSO_4$ were contributed negatively. First order model describing the correlation between eleven factors and levansucrase productivity could be presented as follow:

$$Y_{\text{activity}} = 9.453 + 3.858X_1 + 2.729X_2 + 0.429X_3 + 3.835X_4 + 0.805X_5 - 1.434X_6 - 3.022X_7 - 3.591X_8 - 2.926X_9 + 0.037X_{10} + 1.652X_{11} + 1.873X_{12} - 1.403X_{13} + 2.674X_{14} + 2.425X_{15} \quad (5)$$

Based on the calculated *t*-test and *p*-values (Table 2), it was evident that initial pH, sucrose, yeast extract and KH_2PO_4 were found

to be the most significant variables affecting levansucrase production. Other variables with less significant effect were not included in next optimization experiment, but instead there were used in all trials at their level (+1) level, for the positively contributing variables. According to these results, composition medium consisted of: 10 g of starch moisten with 10 ml of the following medium (g/l): K_2HPO_4, 5; $FeSO_4{\cdot}7H_2O$, 0.001; $CuSO_4{\cdot}7H_2O$, 0.001; $ZnSO_4{\cdot}7H_2O$, 0.001 was used as a plain medium for further investigations.

3.3.1. Fractional factorial design (FFD)

Optimum concentration of most significant culture conditions and medium components (pH, sucrose, yeast extract and KH_2PO_4) showing confidence level 98% and above in Plackett–Burman design for levansucrase production, experiments were performed according to the FFD experimental. Table 3 showed the coded and uncoded levels of three independent variables moreover the observed and predicted levansucrase production was shown. Multiple regression analysis of experimental data gave the following second order polynomial Eq. (6).

$$Y_{\text{activity}} = -87.748 + 163.855X_3 + 15.487X_4 + 2.041X_1^2 + 0.01X_2^2 - 34.035X_3^2 - 2.12X_1X_2 - 10.398X_1X_3 + 1.369X_1X_4 - 0.026X_2X_3 - 0.141X_2X_4 - 3.924X_2X_3 \quad (6)$$

where Y_{activity} is response (levansucrase production) and X_1, X_2, X_3 and X_4 are coded values of test variables (pH, sucrose, yeast extract and KH_2PO_4) respectively. Three-dimensional response surface and two-dimensional contour plots are graphical representations of the regression equation. They are helpful in understanding both main and interaction effects of the factors on the response value. Fig. 2A–D showed response surface and contour plots of pH and yeast extract, pH and KH_2PO_4, pH and sucrose and yeast extract on levansucrase production respectively, keeping other component at fixed zero level.

Regression results from FFD showed that *F*-value (7.586) which implied the model to be significant. Model terms have values of Prob > *F* (0.006) less than 0.05, considered significant. Regression equation obtained after ANOVA indicating that the determination of coefficient (R^2) was calculated as 0.923 for levansucrase productivity which indicated that the statistical model could explain 92.3% of response variability. An overall 4.59 – fold levansucrase productivity increase was being achieved after response surface methodology (RSM) application.

3.3.2. Validation of the model

The validation was carried out under medium optimum conditions predicted by the polynomial model. The experimental levansucrase production of 170 U/g ds was obtained which is closer

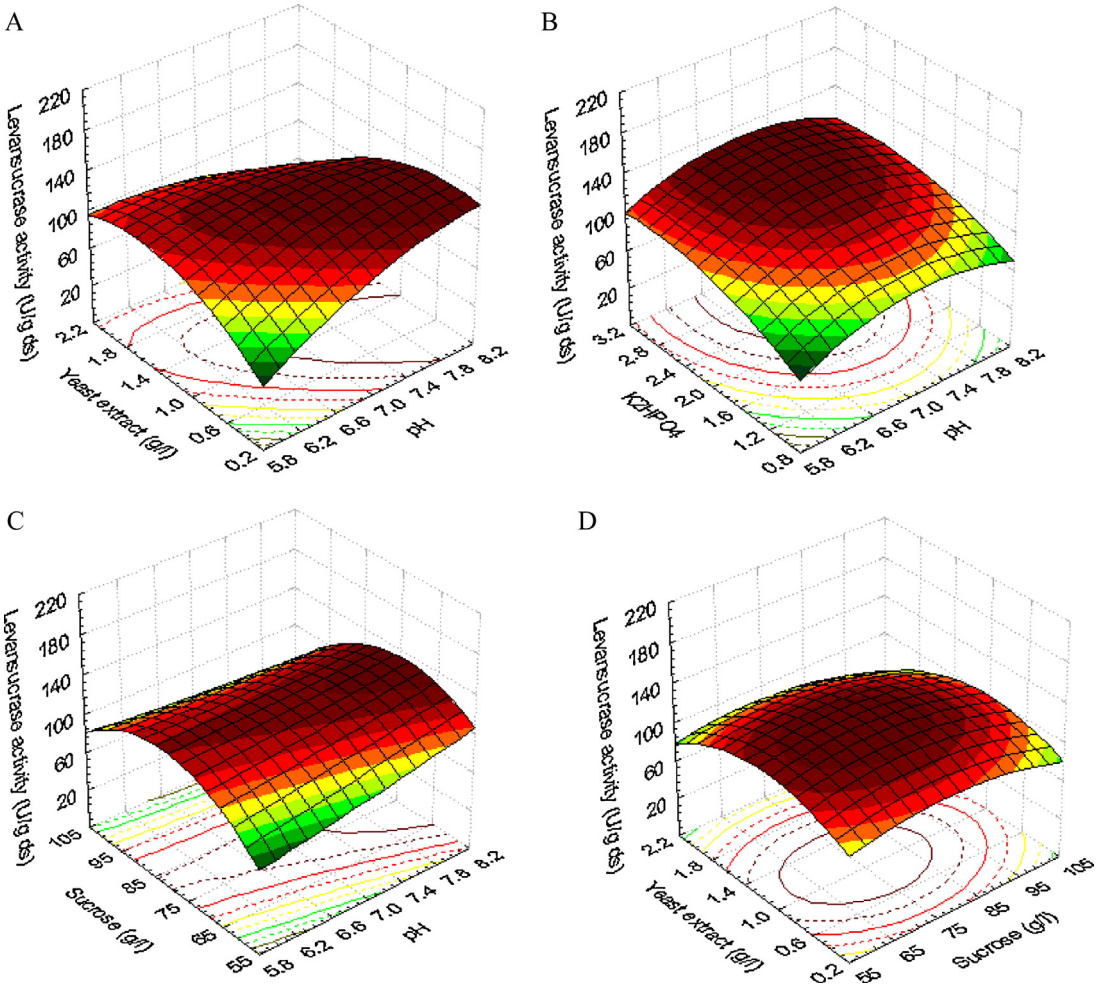

Fig. 2. (A) Response surface plot of levansucrase production by *B. subtilis* NRC1aza showing the Interactive effects of different concentrations of yeast extract and pH at $X_2 = 0$ and $X_4 = 0$. (B) Response surface plot of levansucrase production by *B. subtilis* NRC1aza showing the Interactive effects of different concentrations of $KH_2PO_4$and pH at $X_2 = 0$ and $X_3 = 0$. (C) Response surface plot of levansucrase production by *B. subtilis* NRC1aza showing the Interactive effects of different concentrations of sucrose and pH at $X_3 = 0$ and $X_4 = 0$. (D) Response surface plot of levansucrase production by *B. subtilis* NRC1aza showing the interactive effects of different concentrations of yeast extract and sucrose at $X_1 = 0$ and $X_4 = 0$.

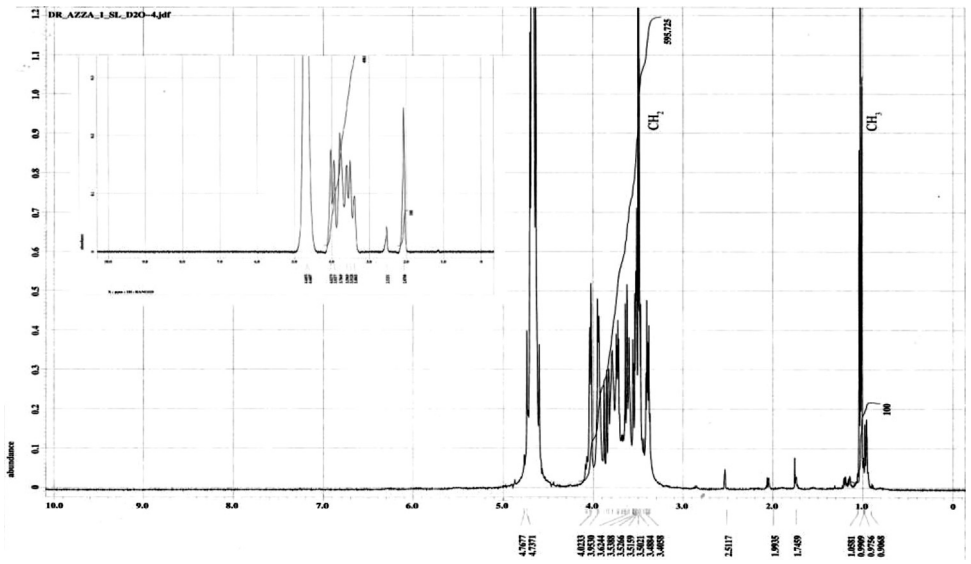

Fig. 3. ^{1}H NMR of levan and quaternized levan (fig. inside) represents the ^{1}H NMR diagram of the levan (fig. outside) represents the ^{1}H NMR diagram of the quaternized levan.

to the predicted levansucrase production of 164 U/ml after 24 h of incubation validating the proposed model. A second order polynomial model was established using fractional factorial design to identify the relationship between the four factors and the levansucrase yield. The final medium components concentrations optimized with RSM were 10 g starch moisten with 10 ml of the following medium (g/l): K_2HPO_4, 5; $FeSO_4{\cdot}7H_2O$, 0.001; $CuSO_4{\cdot}7H_2O$, 0.001; $ZnSO_4\cdot7H_2O$, 0.001 sucrose, 60; yeast extract, 0.5; KH_2PO_4, 3. The initial pH was adjusted to 8.

3.4. LevCR and LevQT characterization

The obtained LevCR had bright white color and crystal form. Its weight was 1 g/l. The LevQT had brown color and sticky form. Paper chromatography investigation indicated that LevCR contained free fructose together with polymerized fructofuranosyl moieties. This finding was supported by ^{1}H NMR (Fig. 3) analysis (in D_2O^{+2} drops of triflouroacetic acid) which revealed the presence of seven proton resonances system of fructoparnosyl moieties [δ pp (m, 3.40–4.2)]. The multiplication of each of these resonances is obviously due to the polymeric nature of the levQT. ^{1}H NMR spectrum of LevQT though was similar to that of LevCR yet a distinction could be made due to the presence of the ethylamine proton resonances attached to the furonzyl moieties present in the spectrum of LevQT [δ ppm: 1 Ct, $j = 8H_2$, CH_3); 3.8 (δ, $j = 8H_2$, (CH_2))].

3.5. Biological activity of LevCR and LevQT

3.5.1. In vitro cytotoxic and antitumor effect of levan and quaternized levan

A preliminary test for the effect of LevCR and LevQT on Ehrlich ascites carcinoma cell line growth, result showed that LevQT inhibited cells growth and had anticancer activity at different concentration (200, 400, 600, 800, and 1000 μg/ml). The death of cells reached to 80% at 800 μg/ml concentration, no more inhibition was detected with increase in levQT concentration, while LevCR was less effective, it inhibited the viability of carcinoma cell line by about 30% inhibition with 1000 μg/ml concentration (data not shown).

3.5.2. In vitro antiproliferative activity

The antiproliferative activity of LevCR and LevQT was evaluated against human breast cancer cell line MCF-7; human liver cancer cell line HepG2 and human lung cancer cell line A549 using Sulforhodamie B (SRB) colorimetric assay, in comparison with cisplatin as reference drug.

The antiproliferative activities were expressed by median growth inhibitory concentration (IC_{50}) and provided in Table 4. The results showed that LevCR and LevQT displayed potent growth inhibitory activity against two tested cell lines only (MCF-7 and HepG2), with no effect on A549 cell line growth, in particular LevQT was found to be near potent and efficacious compared to cisplatin in both MCF-7 and HepG2. Both MCF-7 and HepG2 cell lines showed a normal growth in our culture system. DMSO did not seem to have any noticeable effect on cellular growth.

Table 4 showed that IC_{50} of LevCR and LevQT for MCF-7 cell line was 43.0 and 30.0 μg/ml respectively, it is clear that IC_{50} of LevQT is closed to the value of the reference drug, cisplatin (18.0 μg/ml). Similarly, LevCR and LevQT for HepG2 cell line was 64.0 and 44.0 μg/ml respectively, also IC_{50} of LevQT is closed to cisplatin (27.0 μg/ml).

3.5.3. Biochemical assays

To elucidate the mechanism by which LevCR and LevQT exert their antitumor activities, these compounds were selected to estimate their ability to induce oxidative stress in two human cancer cells (MCF-7 and HepG2). We estimated the activities of the free-radical-metabolizing enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) as well as the levels of oxidative stress parameters including hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) in cells treated with LevCR and LevQT. Additionally, the effect of these compounds on the levels of total protein and nucleic acids was determined.

As shown in Table 5, in general treatment of the cells with different LevCR and LevQT or cisplatin (at the 1/10 of IC_{50} values) resulted in a significant increase in the activity of SOD and level of H_2O_2 higher than those of control (DMSO treated), accompanied with a significant decrease in the activity of CAT and GSH-Px, and depletion in GSH level, indicating an increase in the cellular levels of reactive oxygen species. Moreover, results in Table 5 illustrated that, treatment with these compounds led to significant increase in the level of NO accompanied with significant decrease in the levels of proteins, RNA and DNA.

Table 5
Effect of treatment with the prepared LevCR and LevQT on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), as well as the levels of reduced glutathione (GSH), hydrogen peroxide (H_2O_2), protein, nucleic acids and nitric oxide (NO) in MCF-7and HepG2 treated cells.

Cell line	Compound	SOD U/mg protein	CAT U/mg protein	GSH-Px U/mg protein	GSH nmol/mg protein	H_2O_2 nmol/mg protein	Protein ($\mu g/10^6$ cells)	RNA ($\mu g/10^6$ cells)	DNA ($\mu g/10^6$ cells)	NO μmol/mg protein
MCF-7	Control (DMSO)	42.60 ± 4.5	7.60 ± 0.70	9.30 ± 1.00	40.00 ± 5.00	15.70 ± 1.60	125.50 ± 12.00	18.20 ± 1.90	11.50 ± 1.10	2.20 ± 0.26
	Cisplatin	136.10 ± 14.4[a]	2.96 ± 0.22[a]	3.70 ± 0.40[a]	21.60 ± 2.40[a]	67.50 ± 7.00[a]	35.70 ± 3.75[a]	4.80 ± 0.46[a]	3.70 ± 0.32[a]	6.20 ± 0.60[a]
	LevCR	106.30 ± 12.0[a]	3.60 ± 0.40[a]	4.80 ± 0.60[a]	23. 50 ± 2.20[a]	45.40 ± 3.65[a,b]	40.60 ± 4.00[a]	5.20 ± 0.28[a]	5.00 ± 0.46[a,b]	5.60 ± 0.60[a]
	LevQT	130.00 ± 13.3[a]	2.30 ± 0.24[a]	3.85 ± 0.62[a]	17. 80 ± 0.20[a,b]	63.30 ± 4.80[a]	25.50 ± 2.60[a]	3.50 ± 0.30[a,b]	3.00 ± 0.35[a]	6.00 ± 0.55[a]
HepG2	Control (DMSO)	30.56 ± 3.7	8.60 ± 0.87	13.20 ± 1.30	45.80 ± 4.70	11.50 ± 1.5	170.50 ± 16.00	21.70 ± 1.95	16.50 ± 1.80	5.20 ± 0.46
	Cisplatin	280.20 ± 30.0[a]	2.30 ± 0.16[a]	3.40 ± 0.28[a]	11.20 ± 1.00[a]	77.80 ± 8.00[a]	60.30 ± 6.80[a]	5.20 ± 0.45[a]	5.80 ± 0.48[a]	2.50 ± 0.30[a]
	LevCR	90.60 ± 9.2[a,b]	4.85 ± 0.60[a,b]	6.30 ± 0.55[b]	19.11 ± 1.30[a]	45.60 ± 4.40[a,b]	75.80 ± 8.00[a]	8.80 ± 0.78[a,b]	6.60 ± 0.65[a]	3.30 ± 0.32[a]
	LevQT	180.00 ± 12.2[a,b]	3.30 ± 0.36[a,b]	4.80 ± 0.50[a]	13. 20 ± 1.20[a]	60.80 ± 5.60[a]	55.30 ± 5.60[a]	6.00 ± 0.66[a,b]	5.30 ± 0.60[a]	2.80 ± 0.70[a]

Data are expressed as means ± S.E. of three separate experiments. a and b is significant difference from control and cisplatin groups respectively at ($p < 0.05$)using one way ANOVA test followed by Student's *t*-test.

3.5.4. Fibrinolytic activity analysis of levan and quaternized levan

In vitro fibrinolytic activity analysis of LevCR and LevQT was done and compared with standard fibrinolytic compound heamoclar (pentosan sulfuric polyester, product of ClinMidy, Paris). The results showed that the LevCR exhibited 80% fibrinolytic activity equivalent to double of the effect (40%) caused by the same amount of standard heamoclar "pentosan sulfuric polyester" at the same concentration. On the other hand, the LevQT showed 50% fibrinolytic activity on plasma clot (data not shown).

4. Discussion

Solid-state fermentation (SSF) has emerged as a potential technology for microbial metabolites production. Selection of ideal substrate/support materials for enzyme production in a solid-state fermentation process depends on several factors, mainly related with cost and availability of substrate material, and thus may involve screening of several substrates (Pandey et al., 2000). Levansucrase is considered one of the most important enzymes play a role in public health for human in the last decade. Its importance comes from its ability to yield one of the most essential polysaccharide called levan. The production of levansucrase under submerged culture has mentioned in extensive reports (Ben Ammar et al., 2002; Kojima, Saito, Iizuka, Minamiura, & Ono, 1993), but till now no obvious studied concerned levansucrase production and levan yield in SSF. Within this context, this study tries to introduce a new aspect in *B. subtilis NRC1aza* levansucrase production under SSF.

Previously many authors reported in *B. subtilis* as levansucrase producers (Abdel-Fattah, Mahmoud, & Esawy, 2005; Abdel-Fattah et al., 2012; Esawy et al., 2011). Among different crude and waste materials tested commercial starch gave maximum enzyme productivity and was chosen as SSF support. It was reported in *Bacillus* sp. TH4. levansucrase produced in the presence of 200 g/l starch (Ben Ammar et al., 2002). In addition, starch is an inexpensive and abundantly available raw material and provides the organism with an environment closer to its natural habitat.

Combination of Plackett–Burman design and fractional factorial design was shown to be effective and reliable in selecting the statistically significant factors and finding the optimal concentrations of those factors for levansucrase production by *B. subtilis* NRC1aza in SSF as calculated by Box, Hunter, and Hunter (1978). First, Plackett–Burman design was applied to reflect the relative importance of various medium components. Variables with confidence levels greater than 90% ($p < 0.10$) were considered as significant. From the regression analysis of the variables, and based on evaluation of statistical significance *t*-value and *p*-value of the model pH, sucrose, yeast extract, and K_2HPO_4 were found to display positive effects on levansucrase production. The four factors were previously reported as the main medium constituents affecting the levansucrase production (Abdel-Fattah, Mahmoud, et al., 2005; Esawy et al., 2011). Similar result was obtained by optimization of *Paenibacillus polymyxa* EJS-3 exopolysaccharides (EPS) production using sequential statistical optimization process, the results confirmed that sucrose; yeast extract and $CaCl_2$ influenced significantly its yield (Liu et al., 2010). Levansucrase productivity achieved 4.59 fold increase was being achieved after RSM application. This reflected the necessity and optimization process value. Levansucrase production in experimental results was very close to predicted results. The determination coefficient R^2 of the model was, 0.923. Liu et al. (2010) reported that the high degree of similarity between predicted and experimental values also reflected the accuracy and applicability of RSM for medium optimization to attain maximum EPS production. All previous results indicated that levansucrase productivity could be enhanced by fermentation conditions optimization.

NMR spectra of levan confirmed the polymerized fructofuranosyl moieties nature for LevCR. On contrary, the *B. subtilis NRC1aza* levan isolated in submerged culture was 3:1 fructose and glucose respectively. This result referred to the importance of cultivation conditions in affecting the levan structure. Also the results indicated the configuration in LevCR occurred by quaternization process.

Scientists' studies about cancer therapy have focused on ideal drug being ineffective or minimally effective for normal cells. At this point, the usage of natural sources is thought to have a great value for cancer control and programs' destruction (Suffiness & Pezzuto, 1991).

To our knowledge no previous reports dealt with studying the anticancer activity of quaternized levan and its derivative. Accordingly, one of the most our important goal was anticancer activity investigation for LevCR and LevQT against Ehrlich ascites carcinoma cell line as a model of animal cancer and against different human cancer cell lines including MCF-7, HepG2 and A549.

In vitro preliminary evaluation of the cytotoxic activity of LevCR and LevQT on Ehrlich ascites carcinoma cell line viability revealed that quaternized levan was more superior to LevCR. Increasing LevCR and LevQT concentration caused cell viability decrease. In addition, the *in vitro* results against different human cancer cell lines including MCF-7, HepG2 and A549 showed that both LevCR and LevQT had significant anticancer activity against both MCF-7 and HepG2. Also, results indicated that LevQT was highly effective in decreasing cancer cells viability in compared to LevCR. Based on LevQT NMR data, this result could be attributed to formation of new polymer or the methyl group. This result was in accordance with Liu et al. (2012) who reported that, acetylated levan, phosphorylated levan and benzylated levan exhibited higher anti-proliferative activity against human gastric cancer BGC-823 cells *in vitro* than native EPS. Enhanced activities of derivatives were probably due to introduction of acetyl, benzyl, or phosphoryl groups into EPS-1 molecules that increased electron-donating ability and affinity with immune cells receptors (Liu et al., 2012).

Antitumor activity of these compounds was accompanied by high activity of SOD with subsequent increase in H_2O_2 production. The produced H_2O_2 should be rapidly removed through activation of CAT and GSH-Px. Results showed that activities of CAT, GSH-Px and reduced GSH level were lowered in groups treated with prepared compounds compared to control cells. Consequently, excess H_2O_2 produced in tumor cells cannot be removed. In other words, the accumulation of H_2O_2 and other free radicals in tumor cells could be partially caused of tumor cell killing. Changes in free-radical-metabolizing enzymes activities and oxidative stress parameters of LevQT exhibited higher activity than LevCR which is in accordance with the order of antiproliferative activity of these compounds. LevQT was the most potent antitumor, which resulted in highest SOD activity and H_2O_2. Also, low activities of CAT and GSH-Px as well as GSH level than other compound. Consistency between antiproliferative activity and biochemical assay results indicated that the antitumor effect of the present compounds could be exerted at least partly by production of ROS. Moreover, Bienvenu, Caron, Gasparutto, and Kergonou (1992) reported that most chemotherapeutic agents cause cells to over generate ROS and thus, are capable of inducing apoptosis, and causing oxidative damage to DNA and proteins. The cascade of signals mediating apoptosis often involves a ROS intermediate messenger, and ROS can short circuit the pathway, by passing the need for upstream signals for cell suicide. Latter, Huang et al. (2000) reported that regulation of free radical-producing agents may also have important clinical applications. This mechanism for effects of ROS generating anticancer agents is only beginning to be understood, as previously the mechanism of most anticancer agents was believed to be due mainly to direct interaction with DNA and interference with DNA regulatory machinery (*e.g.*, topoisomerases and helicases) and to initiation of DNA damage *via* production of ROS (Gewirtz, 1999). Results referred to significant increase in the level of NO in presence of the LevCR and LevQT. These results were in accordance with that reported by Schepetkin and Quinn (2006) who mentioned that polysaccharides isolated from algae have been reported to modify macrophage activity by inducing the production of cytokines and nitric oxide. There is a growing body of evidence indicating that NO is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria and therefore make it more permeable (Brüne, 2005). In addition, the elevated level of NO was accompanied with depletion in the levels of total protein and nucleic acids compared to control. This could be explained by several cytotoxic effects that include reaction of NO with proteins and nucleic acids. The main targets of NO in proteins are the thiol group (Molina et al., 1992) and iron of active sites (Hibbs, Taintor, Vavrin, & Rachlin, 1988). In nucleus, NO has been shown to cause gene mutation (Juedes & Wogan, 1996), to inhibit DNA repair enzymes (Lepoivre, Fieschi, Coves, Thelander, & Fontecave, 1991), and to mediate DNA strand breaks (Fehsel et al., 1993).

Also, the findings presented in this study demonstrated the capacity of LevCR to liquefy rapidly clotted fibrin of normal human plasma, but LevQT had no effect. This result introduced new approach in levan applications. Fibrinolytic activity of polysaccharides was reported by many authors (Al-Nahas, Darwish, Ali, & Amin, 2011).

5. Conclusion

This study tried to introduce a new aspect in *B. subtilis NRC1aza* levansucrase production and levan yield under SSF. The yielded LevCR characterized by high purity and its derivative LevQT was highly effective as anticancer agent against MCF-7 and HepG2 cancer cell lines in compared to LevCR. Finally, LevCR showed great fibrinolytic activity. To our knowledge none reported in levan fibrinolytic activity and in quaternized levan.

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