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Levansucrase optimization using solid state fermentation and levan biological activities studies

Mona A. Esawy[a], Azza M. Abdel-Fattah[a], Mamdouh M. Ali[b], Wafaa A. Helmy[a], Bassem M. Salama[a], Hanan A.A. Taie[c], Amal M. Hashem[a], Ghada E.A. Awad[a,*]

[a] Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Giza, Egypt
[b] Biochemistry Department, Division of Genetic Engineering and Biotechnology, National Research Centre, Dokki, Giza, Egypt
[c] Department of Plant Biochemistry, National Research Centre, Dokki, Giza, Egypt

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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

* Corresponding author. Tel.: +202 33335933; fax: +202 33370931.
E-mail addresses: ghadaawad18@yahoo.com, ghadaawad18@gmail.com (G.E.A. Awad).

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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