Solid-State Fermentation for The Production of Dextran from *Saccharomyces cerevisiae* and Its Cytotoxic Effects

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Abstract: Maximum yield of dextran was obtained when using ground date seeds. Different concentrations of date seeds were applied and the highest dextran production was achieved at 6 g/flask. Extraction of dextran was carried out using ethanol. The molecular weight of the purified dextran was 67 kDa by GPC. Spectral analysis showed that dextran contains D-glucose units in a linear chain with consecutive α-(1,6) linkages. The melting temperature (T_m) was 70.56°C, and the value of ΔH was -290.57 mJ, as determined by DSC analysis. The TGA clearly showed the thermal stability of purified dextran. The analysis showed three stages of degradation process. An initial loss of about 0.577 mg (2.17%) weight occurred at 30-75°C, second loss of about 0.822 mg (3.1%) weight occurred at 75-125°C and the third loss of about 0.427 mg (1.61%) weight occurred at 125-175°C. Surface morphology of dextran using scanning electron microscopy showed dextran has a crystalline form which is attributed to the presence of hydroxyl groups which increase crystallinity of dextran, also, dextran showed a compact structure characterized by transversal arrangement which reflects brittleness of dextran and seems to have a porous structure. The cytotoxicity assays on human normal melanocytes (HFB 4) revealed no toxic effect. However, a clear decrease in cell survival was observed in case of human liver carcinoma (HEPG 2) and cervical carcinoma (HELA) tumor cell lines.

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1. Introduction

In a time where prices are increasing in wide steps, search for cheap industrial raw materials has evolved. The use of industrial or agricultural wastes could save disposal efforts and pollution hazards. Solid-state fermentation (SSF) is a means for the microbial conversion of lignocellulosic biomass. SSF is defined as the growth of microorganisms in the absence or near absence of free water with inert or natural substrates as solid support (Pandey *et al*., 1999). SSF is an advantageous method for degrading lignin and improving the digestibility of agricultural straw. Compared with submerged fermentation, SSF possesses many advantages such as low effluent generation, requirement for simpler fermentation equipment, and the direct applicability of the fermented product for feeding (Yang *et al*., 2001). During SSF, microorganisms produce lignocellulolytic enzymes, degrade components of the cell wall, and synthesize microbial proteins. A number of microorganisms, mainly white rot fungi, have been used for producing microbial proteins and lignocellulolytic enzymes by SSF from different agricultural residues (Bisaria *et al*., 1997; Niladevi *et al*., 2007; Arora and Sharma, 2009; Li *et al*., 2011).

Dextrans are synthesized by dextransucrase, an extracellular enzyme secreted by lactic acid bacteria *viz*, *Leuconostoc*, *Lactobacillus* and *Streptococcus* (Katina *et al*., 2009), *Pediococcus* (Patel *et al*., 2010). Dextrans are glucose polymers (Thoren, 1981), which structurally and predominantly consist of linear α-1,6-glucosidic linkage with some degree of branching *via* α-1,3 linkage (Larsen, 1989). In addition to high water solubility, dextran polymers are stable under mild acidic and basic conditions (Schacht, 1987) and contain large number of hydroxyl groups for conjugation. These suitable physicochemical characteristics along with the low cost and a history of clinical use make dextrans attractive for use in pharmaceutical, food, agricultural and fine chemical industries (Naessens *et al*., 2005; Purama and Goyal, 2005) and most of all as potential macromolecular drug or protein carriers (Mehvar, 2001).

A macromolecular drug carrier is typically composed of a macromolecule covalently linked to a therapeutic agent, and it targets solid tumors either passively (*via* its molecular weight and charge) or actively (*via* a specific affinity [e.g., an antibody] or stimulus) (Ringsdorf, 1975; Tomlinson, 1985; Duncan, 2003). In addition to the enhanced permeability and retention effect, macromolecular drug carriers have a longer plasma half-life, reduced toxicity in normal tissue, and higher activity against multiple drug-resistant cell lines than typical chemotherapeutic agents, and they have the ability to

increase solubility of poorly soluble drugs in plasma (Kopecek *et al*., 2000; Duncan, 2003). Because of these characteristics, macromolecular drug carriers coupled to a low molecular drug often have higher anticancer efficacy than the low molecular weight drug alone (Kopecek *et al*., 2000; Duncan, 2003).

The objective of this work was firstly, to use of date seeds waste, thus saving efforts in its disposal, secondly, production of dextran, a polysaccharide with wide and important industrial and pharmaceutical applications, and finally, studying the cytotoxic effect of the produced dextran against human normal and tumor cell lines.

2. Materials and Methods

Microorganism and culture conditions

S. cerevisiae NRRL Y-1534 (CBS 1429) was kindly provided from National Regional Research Laboratory, United States Department of Agriculture, Agricultural Research Services, USA to Prof. Tarek A. A. Moussa and maintained on YM medium which contains (g/l): yeast extract 3 g, malt extract 3 g, peptone 5 g and dextrose 10 g.

Preparation of agricultural wastes

The date seeds were washed and left to air dry. The air dried seeds were ground to fine particles using blender. The rice and wheat straws were collected and left to air dry then ground. The wheat bran was bought from the market.

Production of dextran from agricultural wastes using *S. cerevisiae*

S. cerevisiae was cultured on solid medium composed of: agricultural wastes, 4 g; yeast extract, 0.3 g; K_2HPO_4, 0.5g, all components were mixed in 500 ml flasks. The culture was incubated at 25°C for 48 hrs. The produced dextran was purified and weighed.

Production of dextran from different weights of date seeds wastes using *S. cerevisiae*

S. cerevisiae was cultured on solid medium composed of: yeast extract, 0.3 g; K_2HPO_4, 0.5 g, all components were mixed in 500 ml flasks. Date seeds waste was added to the flasks in different weights (2, 4, 6, 8 or 10 g). After incubation at 25°C for 48 hrs, dextran was purified and weighed.

Extraction and purification of dextran

100 ml of dist. water was added to the whole flask which was shaken well and then the contents were filtered. These steps were repeated twice and the filtrates were collected and combined. Extraction of dextran was carried out according to Behravan *et al.* (2003); equal volume of ethanol was added, kept at 4°C for 24 hrs. The solution was centrifuged at 5000 rpm. The supernatant was discarded and the precipitate was dissolved in minimal volume of water. Twenty mg of activated charcoal were added to the dissolved precipitate and boiled, then filter. The protein was precipitated by adding amyl alcohol/chloroform, then centrifuge at 5000 rpm and the precipitated protein was removed. Equal volume of cold ethanol (1:1 v/v) was added to the supernatant to precipitate dextran. The obtained dextran was dried in desiccators on anhydrous calcium chloride and weighed.

Gel Permeation Chromatography (GPC)

Average molecular weight of the dextran was determined with gel permeation chromatography coupled with refractive index detection using a TSK G4000 PW_{XL} column. The sample with concentration 60 mg/ml was injected. The mobile phase was 0.02% (w/v) sodium azide in HPLC grade water at a flow rate of 0.5 ml min^{-1}. Fractions (250 µl, each) were collected at room temperature. The column was calibrated with respect to fraction volume using five standard polymers with molecular weights from 25 to 2000 kDa.

Structural Spectroscopy

Purified sample was analyzed by transmittance IR spectroscopy in the form of KBr pellet using a Fourier Transform Infrared spectrometer (FTIR 6100). The ^{1}HNMR spectrum of the sample was recorded on a Varian Mercury VX-300 spectrometer. The purified dextran was dissolved in D_2O (10 mg /ml). ^{1}HNMR was run at a base frequency of 300 MHz.

Differential Scanning Calorimetry (DSC) and Thermogravimetric Analyzer (TGA)

Differential scanning calorimetry analysis was conducted using a DSC-50 Shimadzu Differential Scanning Calorimeter. The sample was tightly sealed in aluminum pans and heated at a constant rate 10°C min^{-1} over a temperature 30-150°C and under nitrogen purge. Thermogravimetric studies were carried out using a TGA-50 Shimadzu.

Scanning Electron Microscopy (SEM)

Morphology of the obtained dextran was examined by an SEM. The dried sample was mounted on the SEM stub with double-sided tape then sputter coated with gold using SPI-Module™ sputter coater (SPI Supplies Division of Structure Probe, Inc.). The sample surface was imaged using a Jeol JSM-5200 scanning microscope operated at 10 kV.

Cytotoxicity Assays

Cytotoxicity of the obtained dextran was tested against the normal human cell line: human normal melanocytes (HFB 4), and the human tumor cell lines: breast carcinoma (MCF 7), liver carcinoma (HEPG 2) and cervical carcinoma (HELA). The method of Skehan *et al.* (1990) was adopted. Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 hrs before treatment with dextran to allow attachment of cell to the wall of the plate. Different

concentrations (1000, 2000, 3000 and 4000 μg/ml) were added to the cell monolayer triplicate wells prepared for each individual dose. Monolayer cells were incubated with dextran for 48 hrs at 37°C and in an atmosphere of 5 % CO_2. After incubation, cells were fixed, washed and stained with Sulfo-Rhodamine-B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured by an ELISA reader (Meter tech. Σ 960, USA). The relation between cell survival (as percentage of control) and dextran concentration was plotted to get the survival curve of each normal or tumor cell line after subjecting to dextran. Cell survival (%) was calculated as follows:

$$\text{Survival (\%)} = (I_t/I_c) \times 100$$

Where, I_t is the color intensity of treated cells and I_c is the color intensity of untreated cells.

3. Rsults and Discussion

Dextran is a very important polysaccharide commonly used in routine medical, microsurgical and laboratory procedures (de Carvalho *et al*., 2001; Rotureau *et al*., 2004; Khalikova *et al*., 2005; Dhaneshwar *et al*., 2006). Dextran derivatives are used for reducing platelet adhesiveness (Siddika *et al*., 1997; Huynh *et al*., 2001), wound healing (Logeart-Avramoglou *et al*., 2002), tumor targeting of gene expression (Hosseinkhani *et al*., 2003) and osmotic pressure control in biological molecules (Rotureau *et al*., 2006) and human enzyme regulation (Ledoux *et al*., 2003). Dextran derivatives have well reported antiproliferative and anti-tumor properties (Bittoun *et al*., 1999). Dextran is receiving attention in the formulation of macromolecular prodrugs of acid sensitive drugs to target them to the colon and in this way, highly sensitive anticancer drugs, such as mitomycin C, can be safely targeted to its site of action (Cheung *et al.*, 2005). Dextran is being used for sustained release studies of several sensitive drug molecules after their covalent attachment (mostly esters) onto polymer backbone (Miyazaki *et al*., 2003).

In this study, different agricultural wastes were used as carbon sources in the fermentation medium of *S. cerevisiae* for the production of dextran. Highest dextran production was obtained from medium with ground date seeds waste compared with other tested agricultural wastes (Table 1). Date seeds wastes were chosen for the production of dextran in the next experiments.

Different weights of ground date seeds were tried. The data presented in Table 2 showed that increasing date seeds weight caused a corresponding increase in dextran production. Maximum dextran yield was achieved when using 6 g of ground date seeds/500 ml flask; however, there was a decrease in the amount of obtained dextran following this concentration.

Probably higher concentrations of date seeds in the fermentation medium showed a substrate inhibitory effect causing decrease in dextran production (Martinez-Espindola and Lopez-Munguia, 1985). Similar results were obtained with sucrose as a substrate for dextran production (Kim *et al*., 2003; Sarwat *et al*., 2008).

There are numerous literature reports on use of gel permeation chromatography (GPC) for the assessment of molecular weights (Nilsson and Nilsson, 1974; Richter *et al*., 1983; Meredith, 1984; Komatsu *et al*., 1993; Van and Daenens, 1993; Mulloy *et al*., 1997; Karmarkar *et al*., 2006). The purity and molecular weight of the produced dextran from *S. cerevisiae* was investigated by GPC analysis. A single and unimodal peak was observed (Fig. 1), which identified the obtained dextran not being contaminated. The peak of dextran was from fractions 22-42 and of the tip of peak was at fraction no. 38 which appears after 9.5 ml (each fraction is 250 μl), so from calibration curve, the molecular weight was about 67 kDa (Fig. 1).

The FT-IR spectra were used to investigate the functional groups of commercial dextrans and pullulans and their nature in terms of their monomeric units and their linkages (Shingel, 2002). Several other repots also supported the use of FT-IR spectral data for the characterization of glucans showing anti-cancer properties (Cao *et al*., 2006; Liu *et al*., 2007). FT-IR spectrum data of the purified dextran is presented in Table 3. The band in the region of 3400 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide (Liu *et al*., 2007; Purama *et al*., 2009; Patel *et al*., 2010). The band in the region of 2930 cm^{-1} was due to C–H stretching vibration and the band in the region of 1639 cm^{-1} was due to carboxyl group (Cao *et al*., 2006; Liu *et al*., 2007). The absorption peaks at 616 and 906 cm^{-1} indicate the existence of α-glycosidic bond. The main characteristic bands found in the spectra of dextran at 1152, 1095 and 1024 cm^{-1} are due to valent vibrations of C–O and C–C bonds and deformational vibrations of the CCH, COH and HCO bonds, this was in context with the results reported by many researchers (Shingel, 2002; Purama *et al*., 2009). The band at 1152 cm^{-1} is assigned to valent vibrations of C–O–C bond and glycosidic bridge. The peak at 1103 cm^{-1} is due to the vibration of the C–O bond at the C-4 position of glucose residue (Shingel, 2002; Purama *et al*., 2009). The presence of peak at 1020 cm^{-1} is due to the great chain flexibility present in dextran around the α-(1,6) glycosidic bonds as shown earlier (Shingel, 2002). FTIR spectra of our samples also

show a band in the region of 750-1300 cm^{-1} that corresponds to the carbohydrates region (Table 3). These wave numbers are within the so-called fingerprint region, where the bands are specific for each polysaccharide, allowing its possible detection (Sen and Erboz, 2010). The FTIR spectra of dextran have been investigated in the range between 4000 and 400 cm^{-1} (Shingel, 2002). FTIR spectral analysis of *S. cerevisiae* NRRL Y-1534 dextran showed that it contains α-(1,6) linkages. This was further confirmed by ^{1}H NMR analysis.

In this study, the ^{1}HNMR spectrum for the purified dextran was carried out. It was reported that the distribution of ^{1}HNMR spectral resonances range between 3 and 6 ppm for different dextrans (Seymour, 1979). The ^{1}HNMR spectral resonances (H-2–H-6) lie in the 3-4 ppm region while the hemiacetal H-1 resonance is in the 4–6 ppm region (Sidebotham, 1974; Majumder and Goyal, 2009). Seymour (1974) assigned a 4.9 ppm resonance for the H-1 of the α-(1,6) linked glucosyl residues of dextran. Likely, in this work a 4.8 ppm resonance could be assigned for the H-1 of the α-(1,6) linked main chain of dextran from *S. cerevisiae.* Purama et al. (2009) found that the ^{1}HNMR spectrum of *Leuconostoc mesenteroides* NRRL B-640 have an H-1 at 4.9 ppm deducing production of highly linear, α-(1,6) linked, water soluble dextran. The dextran produced by *Pediococcus pentosaceus* (Patel *et al*., 2010) showed similar results.

Differential scanning calorimetry (DSC) is a technique that measures the energy difference between a sample and the reference cell as a function of time when they are submitted to a heating program. The technique is useful for polymers, especially hydrogels, because the structural changes accompanied by energetic effects can be followed in DSC curves. As the temperature increases the sample eventually reaches its melting temperature (T_m), which was 70.56°C. The melting process resulted in an endothermic peak in the DSC curve. The enthalpy (ΔH) value was -290.57 mJ (Fig. 2).

The thermogravimetric analysis clearly showed the thermal stability of purified dextran. The analysis showed a three stage of degradation process. An initial loss of about 0.577 mg (2.17%) weight occurred at 30-75°C, second loss of about 0.822 mg (3.1%) weight occurred at 75-125°C and the third loss of about 0.427 mg (1.61%) weight occurred at 125-175°C. Thermal parameters as the melting point and decomposition temperature are useful to engineers who must select polymers to withstand a given thermal environment.

The surface morphology of the purified dextran produced by *S. cerevisiae* was studied. In Fig. 4a, dextran appears in a crystalline form which is attributed to the presence of hydroxyl groups which increase crystallinity of the polymer (Purama *et al*., 2009). Dextran shows a compact structure (Fig. 3b) characterized by transversal arrangement which reflects brittleness of dextran [52]. Meanwhile, dextran seems to have a porous structure (Fig. 3c). The small pore distribution can cause dextran to hold water and thus be used as a texturing agent in food industry. These small pores may also be responsible for the compactness of the polymer and the stability of the gel structure when subjected to external forces and the maintenance of the texture properties during storage (Khan *et al*., 2007; Purama *et al*., 2009).

Table 1. Effect of different agricultural wastes on the production of dextran by *Saccharomyces cerevisiae* on solid-state fermentation

Substrate	Dextran (g/100 ml)
Date seeds	1.092
Rice straw	0.813
Wheat bran	0.880
Wheat straw	0.961

Table 2. Effect of different weights of date seeds wastes on the production of dextran by *Saccharomyces cerevisiae* on solid-state fermentation

Date seeds wt.	Dextran (g/100 ml)
2	1.056
4	1.092
6	1.560
8	1.242
10	1.068

Results of dextran cytotoxicity assay revealed that it has no obvious harmful effect on human normal melanocytes (HFB 4) even after the concentration was raised up to 4000 µg/ml (Fig. 4). It can be deduced that dextran produced by *S. cerevisiae* is a non-toxic compound which can be safely used in biomedical applications including drug delivery. The results are in agreement with the previous findings of De Groot *et al*. (2001). When dextran was tested against the human tumor cell lines: breast carcinoma (MCF 7), it had almost no effect. However, a clear decrease in cell survival was observed in case of liver carcinoma (HEPG 2) and cervical carcinoma (HELA). On the other hand, a dextran from another source (Patel *et al*., 2010) showed no effect on HELA cells at a concentration of 1000 µg/ml. Results in the current study suggest that dextran produced by *S. cerevisiae* could have a potential for investigation for tumor therapy as a polymer-drug conjugate. Dreher *et al*. (2006) studied penetration of macromolecular drug carriers to tumors. Increasing the molecular weight of dextran statistically significantly reduced

its vascular permeability but increased its plasma half-life. Tumor accumulation was maximal for dextrans with molecular weights between 40 and 70 kDa, which lie in the range of clinically available and successful macromolecular drug carriers (Duncan, 2003). Dextran in the present work exhibited a molecular weight of about 67 kDa. It could thus show a potential in the use as a macromolecular drug carrier.

Table 3. FTIR spectrum for the purified dextran produced by *Saccharomyces cerevisiae* on solid-state fermentation

No.	Position	Intensity	Group	Comments
1	3434.6	81.6	ν(OH)	Stretching
2	2926.45	88.37	C-H	-CH stretching of CH_2 and CH_3 groups
3	2859.9	90.05		
4	1740.44	90.46	CHO	
5	1630.52	88.12	δ(HOH), δ(OH), -COO-	asymmetrical stretching of carboxylic groups
6	1433.82	90.21	δ(OH), -COO-	symmetrical stretching of carboxylic groups
7	1331.61	92.22	δ(OH)	
8	1263.15	92.12		
9	1152.26	91.16	C-O-C	glycosidic bridge
10	1095.37	89.88	C-O, C-C	At C4 position of glucose residue
11	1024.98	88.24	α-(1,6) glycosidic bonds	chain flexibility present in dextran around the glycosidic bonds
12	760.78	95.71	γ(OH)	
13	616.07	93.43		Glycosidic bond
14	418.47	93.86		

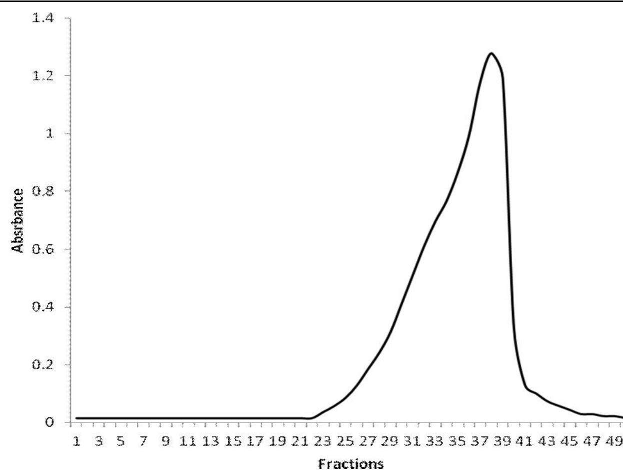


Figure 1: GPC for the produced dextran fractions using TSK G4000 PWXL column produced by *S. cerevisiae* on solid-state fermentation.

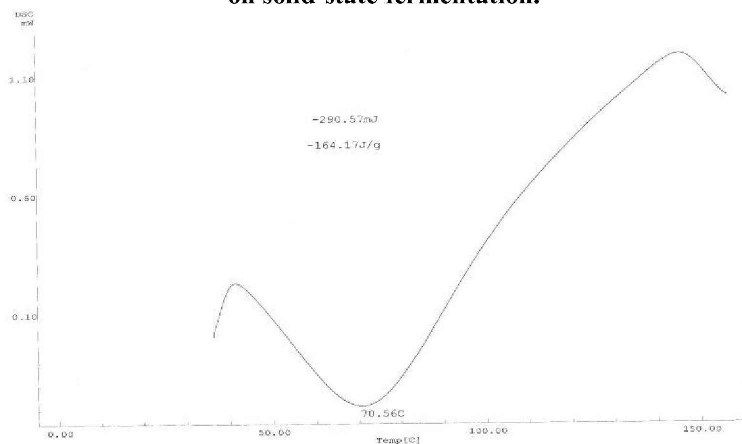


Figure 2: DSC curve for the purified dextran produced by *S. cerevisiae* on solid-state fermentation.

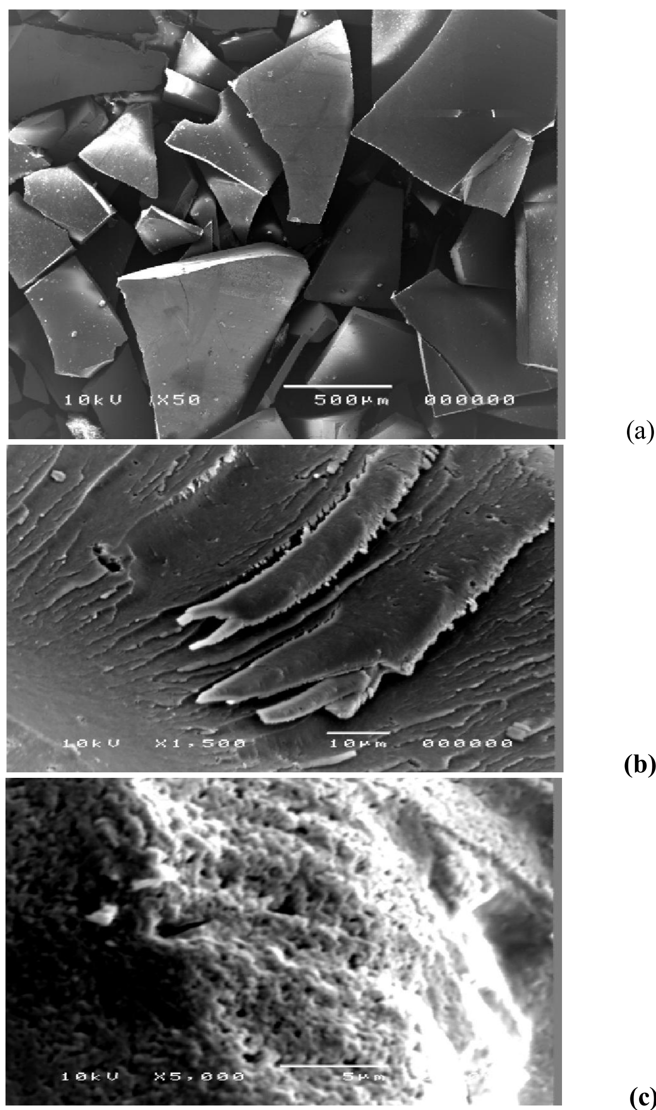
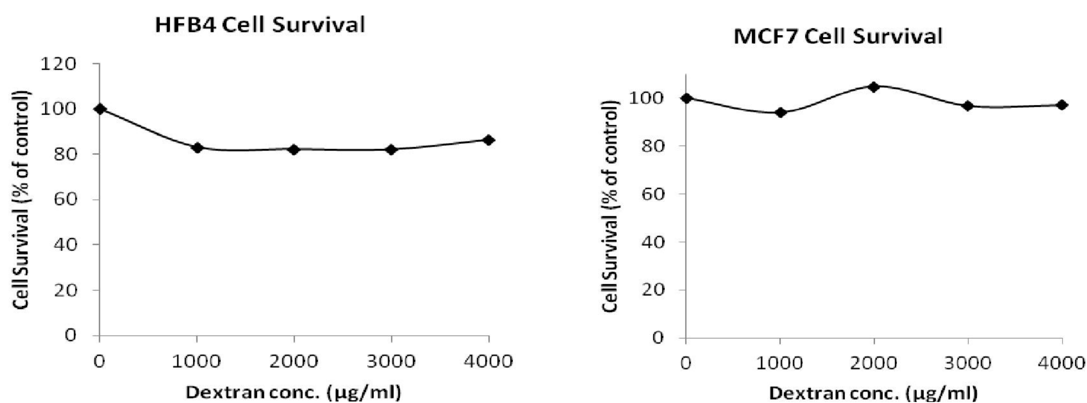


Figure 3: Scanning electron microscopic (SEM) images showing surface morphology of purified dextran from *S. cerevisiae* on solid-state fermentation.



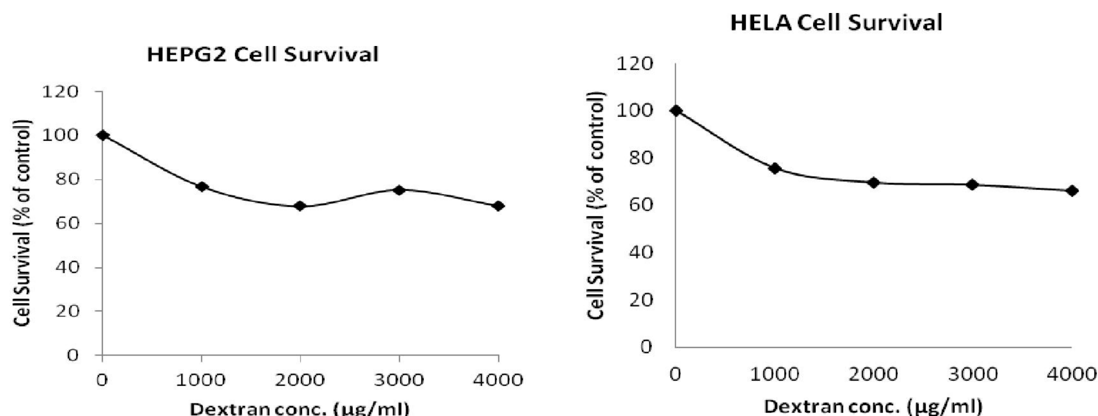


Figure 4: Effect of purified dextran produced by *S. cerevisiae* using solid-state fermentation on cell survival ratio of human normal (HFB 4) and tumor cell lines (MCF 7; breast carcinoma, HEPG 2; liver carcinoma and HELA; cervical carcinoma).

4. Conclusion

Date seeds wastes were successfully utilized by *S. cerevisiae* for production of dextran. Dextran showed thermal stability as well as a crystalline porous structure. Hence, it can be used as a texturing agent in food industry. It had no toxic effect on human normal melanocytes so it can be used safely in biomedical applications. However, it caused a clear decrease in cell survival was observed in case of human liver carcinoma and cervical carcinoma tumor cell lines. The previous result together with the measured molecular weight (67 kDa) renders dextran from *S. cerevisiae* a successful macromolecular drug carrier.

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