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OPTIMIZE OF SOME BIOREACTOR PHYSICAL CONDITIONS AFFECTING ON ACCUMULATION OF ACTIVE COMPOUNDS IN SUSPENSION CULTURES OF EGYPTIAN DATE PALM (SAMANY CULTIVAR).

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ABSTRACT

Date palms (Phoenix dactylifera L., Arecaceae) are one of oldest cultivated plants in the Middle East and North Africa, and used in folk medicine for treatment of various diseases. The concept of this study is investigate the effects of agitation rates (100 to 160 rpm) and aeration (40 to 80 % dissolved oxygen DO) as a stirred tank bioreactor (STB) physical conditions on mass cells proliferation, total phenolic (TP) and total flavonoid (TF) contents. Further, evaluate the antioxidant activity in suspension cultures of Egyptian date palm (Samany cultivar). The highest value of mass cell proliferation recorded with the agitation rate of 120 rpm and 40 (%) of DO. However, the highest values of TP and TF contents 6.45 mg gallic acid equivalents (GAE) per 100 g fresh cells, and 1.12 mg/100g rutin equivalents, respectively recorded with 120 rpm and 60 % of DO. Whereas the antioxidant activities of methanolic extracts were evaluated in vitro using scavenging assays of 1,1-diphenyl-2-picrylhydrazyl(DPPH) radical. Effectiveness scavenging concentration (IC_{50}) on DPPH radical (2.45 mg/l) estimated with 120 rpm and 80 (%) of DO. These results indicated that date palm (Samany cultivar) cell cultures could be physical controlled in bioreactor, further considered a good natural source for active and medicinal compounds production.

KEY WORDS: Date palm, suspension, bioreactor, flavonoids, total phenolic.

Abbreviations

STB (stirred tank bioreactor)

TP (total phenolic)

TF (total flavonoid)

DO (dissolved oxygen)

GAE (gallic acid equivalents)

DPPH (1,1-diphenyl-2-picrylhydrazyl)

INTRODUCTION

Date palm (Phoenix dactylifera L.) is mostly grown in tropical and subtropical regions of North Africa and Southern Asia. It is a major component of agro-ecosystems in hot dry regions providing fruits rich in sugars, minerals, and vitamins (Jameel 2012).

Plants produce several different secondary metabolites, called phytochemicals mostly of them used as pharmaceuticals. In recent years biopharmaceutical industry renewed increased attention in production of health-promoting secondary metabolites using plant cell and tissue cultures. Different efforts to improve their productivity had limited success, especially due to the lack of suitable technologies for such scale-up applications (**Iryna 2008)**. The recent biotechnology boom has triggered increased interest in plant cell cultures. In alternative to wild collection or plant cultivation, the production of useful and valuable secondary metabolites in large bioreactors is an attractive proposal; it should contribute significantly to future attempts to preserve global biodiversity and alleviate associated ecological problems. Moreover, bioreactors have several advantages for the mass cultivation of plant cells: 1) better control for scale-up of cell suspension cultures under defined parameters for the production of bioactive compounds; 2) constant regulation of conditions at various stages of bioreactor operation; 3) easy and efficient handling of culture such as inoculation or harvest; 4) enhanced nutrient uptake by submerged culture conditions which stimulate the multiplication rate and a higher yield of bioactive compound and, 5) large numbers of plantlets are easily produced and can be scaled-up (Fulzele and Heble, 1994; Othmani et al., 2011). The advantages of such processes include the controlled production according to demand and a reduced man work requirement. Plant cells have been grown in different shape bioreactors, however, there are a variety of problems to be solved before this technology can be adopted on a wide scale for the production of useful plant secondary metabolites. There are different factors affecting the culture growth and secondary metabolite production in

bioreactors: the gaseous atmosphere, oxygen supply and CO_2 exchange, pH, minerals, carbohydrates, growth regulators, the liquid medium rheology and cell density (Barbara et al., 2010). Further, the main target of biotechnology is to help convert a country's diverse biological resources into useful products and processes that are accessible to its people for economic development and employment generation (Jain et al., 2011).

The design and operation of a bioreactor are mainly determined by biological needs and engineering requirements, which often include a number of factors: efficient oxygen transfer and mixing, low shear and hydrodynamic forces, effective control of the physico-chemical environment and ease of scale-up Operational parameters such as aeration rate, agitation speed, impeller design, gas mixing and bioreactor configuration are also important because dissolved oxygen (DO) must be transported rapidly to the culture tissues or cells. In general, it is essential that the dissolved oxygen concentration remains above the critical DO_2 level at all the times for optimal cell growth (Leathers et al., 1995, Sajc et al., 2000; Honda et al., 2001).

Antioxidants may be reduce and prevention the risks of chronic diseases, such as atherosclerosis, cancer, cardiovascular, cataract, diabetes, coronary heart diseases, and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (Deepa 2007, Cheel 2007, Podse 2007) as well as inflammation and problems caused by cell and cutaneous aging (Ames 1993) further improve general human health (Iqbal 2007). Some studies have shown that dates have potent anthocyanin, carotenoid, and phenolic compounds (mainly cinnamic acids) and flavonoids (flavones, flavonols and flavanones) and have antioxidant properties (Boudries 2007, Allaith 2008, Biglari 2009).

The interest in antioxidants has been increasing because of their high capacity in scavenging free radicals related to various diseases (Silva et al., 2007). In this respect, phytochemicals from date palm fruits have been shown to possess significant antioxidant capacities that may be associated with lower incidence and lower mortality rates of degenerative diseases in human (Javanmardi et al., 2003). The antioxidant properties of fruits vary depending on their content of phenolic components and vitamins C and E, carotenoids, flavonoids (Saura and Goni, 2006). The main objective of this study was investigate the efficiency of some physical factors such as agitation rate(s) and dissolved oxygen levels affecting on mass cell proliferation , total phenolic and evaluation of antioxidant activity produced from date palm (ِSamany cultivar) cell suspension cultures, cultured on stirred tank reactor.

MATERIALS AND METHODS

Plant material

Female date palm (Phoenix dactylifera L.) offshoots of Egyptian date palm cultivar (Samany) were obtained from Rashed in North Egypt; the offshoots were separated during the fruiting stage from the mother plants. The measurements and parameters of the offshoots were 120-150 cm in height, 35 cm in diameter and 50-55 kg. in weight. These offshoots were used as mother plant materials for initiation of in vitro cultures.

Sterilization

Sterilization of the obtained shoot tip was carried out according the method described by Taha et al. (2012).

Nutrient media and callus production

Explants of sterilized primary basal leaves were excised from the base of shoot tips, further cultured on modified solidified Murashige and Skoog (1962) nutrient medium (MS) as described by Taha et al. (2010). The pH of the culture medium was adjusted to 5.7 with 0.1 M NaOH or 0.1 M HCl before adding phytagel. The culture medium was dispensed into 150 ml jars, each containing 40 ml and autoclaved at 121°C ± 1 for 20 min. Cultures were incubated in darkness in a growth chamber at a constant temperature of 28°C ± 1 then incubated under light condition (2000 Lux) from cool white fluorescent lamps, and subcultured every 6 weeks on fresh new medium. After three subcultures, white calli were initiated and observed.

Cells achievement

Calli were homogenized and re-suspended in an agitated liquid MS medium containing 1 mg/l 2,4-D + 1 mg/l 2iP + 3mg/l NAA according to the best results obtained
by **Taha et al. (2010).**

Specification and operation of bioreactor

Available 2-L turbine stirred tank bioreactor (STB) of the National Research Centre (NRC) was used with a working volume of 1.5-1.7 L (B. Braun, Biotech, International, Germany). The culture was aerated through a stainless steel spurger. The flow rate was set up according to the type of experiment and maintained at the normal level with a mass flow control system until the end of the culture period. Two six-bladed turbine impellers (D=45 mm) were used for mixing at different rotation speeds (rpm). The temperature was maintained at 26°C with a

thermostatic outlet spongy sheet surrounding the vessel. Aeration was performed by filtered sterile air at the rate of 0.5 l/min. Dissolved oxygen concentrations were measured with a sterilizable oxygen electrode (Ingold). Dissolved oxygen concentration was monitored with a sterilizable pO_2 electrode to maintain different levels of dissolved oxygen concentrations in the bioreactor broth, with the inlet air dosed by a mass flow controller connected with software and pO_2 electrode. The bioreactor was inoculated with one part of suspension culture and five parts of medium, and the cell cultures were kept at 25°C. Incorporated MS nutrient medium containing cell lines were introduced into a glass tank bioreactor under sterilized air condition. The physical parameters such as agitation rates (100,120,140 and 160 rpm) and aeration (40,60 and 80 % DO) affecting on mass cell culture and achievement of total phenolic, total flavonoids further, evaluation of antioxidant activity in lyophilized suspension culture of Samany date palm cultivar were investigated, as follows:

Measurement of cell growth parameters

The fresh and dry weights (w/v) were determined using a sampling unit of suspension culture (2 ml) every 2 days for 2 weeks. Results were expressed at the end of the 15 days.

Chemicals and reagents

Solvents and reagents used in the experiments were of the highest purity and purchased from Sigma.

Sample preparation and extraction

All Samany date palm cell lines were lyophilized under completely darkness for completely dryness, further milled into a fine powder. The phenolics were extracted and isolated according to described method by Djeridane et al. (2006 a). Five grams of fine powder macerated in 25 ml methanol: water (80:20, v/v) for 24 h at room temperature. The crude preparation was filtered, and the residue re-extracted twice with 50 ml of the same hydro-alcoholic solvent for 24 h at room temperature. The extract was filtered. The filtrates of hydro-alcoholic were combined. After removing the alcohol under vacuum at 40 °C, the phenolic compounds were extracted three with ethyl acetate (1:1, v/v) in the presence of an aqueous solution containing 20% ammonium sulphate and 2% of orthophosphoric acid solution. The three organic phases were combined; the residual water in the ethyl acetate was eliminated with anhydrous sodium sulphate, and then evaporated to dryness using a rotary evaporator. The extracted phenolics were dissolved in 20 ml of methanol and then filtered using filter paper. Methanolic solutions of phenolic were kept frozen until analysis.

Determination of total phenolic content (TPC).

The amounts of TPC in lyophilized cell line extracts were determined using the described method by Singleton and Rossi (1965). Briefly, reaction mixture contained one 100 µl of methanolic extracts (three replicates), 500 µl freshly prepared dilute (1:10) Folin–Ciocalteu reagent, and 2 ml of Na_2CO_3 (20% w/v). Mixtures were shaken and left to stand at room temperature for 30 min before measuring the absorbance at 760 nm using a spectrophotometer (UV–visible spectrophotometer; Shimadzu). The TPC was determined colorimetric through standard gallic acid curve and expressed as milligrams of gallic acid equivalents per 100 g of dry weight cell lines material (GAE/100 g dw).

Determination of total flavonoid contents (TFC).

TFC was determined according to the aluminum chloride colorimetric method described by Chang et al. (2002) based on the method of Woisky and Salatino (1998) and results expressed as mg of rutin equivalents per 100 g of dry weight (RE/100 g dw). This method is based on the quantification of yellow color produced by the interaction of flavonoids with $AlCl_3$ reagent. To 1 ml of each sample (three replicates), 1 ml of 10% (w/v) $AlCl_3$ in methanol solution and 1 ml of (0.1 N) sodium acetate solution were added and incubated for 40 min in the obscurity at the room temperature. The absorbance of all samples was measured at 410 nm (UV–visible spectrophotometer; Shimadzu).

Antioxidant activity

Radical scavenging activity of Samany cell line extracts against stable DPPH (2-diphenyl-2-picrylhydrazyl hydrate) was determined using the method of Brand-Williams et al. (1995) modified by Djeridane et al. (2006 b). This method depend on the measuring of changes in color (from deep-violet to light yellow) at 517 nm on a UV–visible light spectrophotometer. Solution of DPPH in methanol (500 mM) was prepared daily, before the measurements. Various concentrations of 1 ml of sample solution diluted in Tris buffer solution (100 mM; pH 7.4) were added to 1 ml of the DPPH as radical solution. The mixture was then shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The decrease in absorbance was measured at 517 nm. Absorption of a blank sample containing the same amount of buffer and DPPH solution was prepared and measured daily. The antioxidant activity of the different cell line extracts were expressed as an IC_{50} value defined as the concentration (mg/l) of the extract that inhibited the formation of DPPH radicals by 50%.

Statistical analyses

All experiments were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1980).

RESULTS

Efficiency of STB physical parameters on mass cell production.

In this experiments ~ fifty (g) of fresh and healthy Samany date palm cell line were inoculated to the available STB (2 L) and augmented with MS modified nutrient medium as described by Taha et al.(2012). The effect of some physical conditions such as agitation rates (100,120,140 or 160 rpm) in combination with aeration (40,60 and 80 % DO) on fresh and dry weights (g/ run) of different cell lines were recorded individually and sampling every 2 days for 15 days. Illustrated data in Figs. (1and 2) clearly show that the highest value of mass cell fresh and dry weights 286.15 and 24.12, (g/run) were recorded with aeration of 40 % DO in combination with agitation rate of 120 rpm, respectively. It showed be mentioned that increasing of either agitation rates or dissolved oxygen reduced of cell fresh or dry weights consequently. The R^2 of logarithmic trend line of aeration (40 % DO) was 0.160.

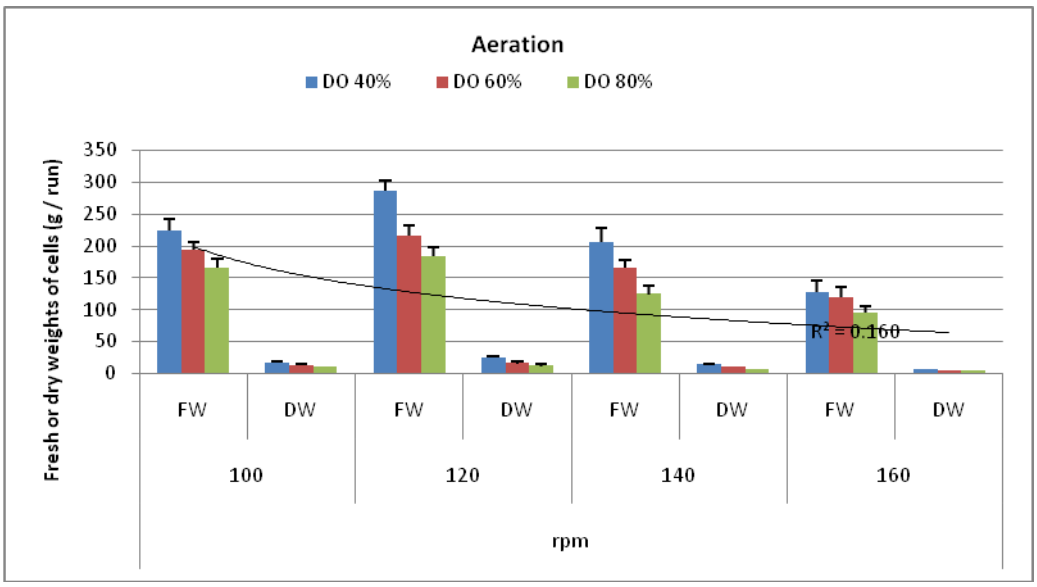

Fig. (1). Effect of different agitation (rpm) and aeration levels as percentage of DO on fresh and dry weights of Samany date palm cell production (g/run) cultured and incubated in bioreactor (STB) at 28 ± 1°C under light condition (2000 Lux) for 2 weeks.

Fig. (2). B-Braun Biotechnology International Stirred Tank Bioreactor (2L).

Determination of TP mg galic acid (GAE)/ 100 g dw and TF mg rutin equivalents (RE)/ 100 g dw compounds in different cell lines of Samany date palm.

In this experiment the TP mg GAE/100g and TF compounds mg RE/ 100 g of lyophilized and dryness Samany date palm cell lines were determined in the previous experiments. Illustrated data in Fig.(3) stated that the effect of different agitation rates (rpm) in combination with different percentage of DO on accumulation rate of TP and TF compounds in different cell lines after the time run duration (2 weeks). The highest values of total phenolic and total flavonoid compounds 6.45 mg/ GAE/ g dw and 1.12 mg/100 g RE were recorded with 120 (rpm) and aeration 60 % (DO), respectively. However the lowest accumulation rates of TP and TF 5.65 mg GAE/100g dw and 0.74 mg RE/ 100 g dw were recorded with 160 (rpm) and aeration 40 % (DO), respectively. The R^2 of logarithmic trend line of aeration of 60 % DO was 0.055.

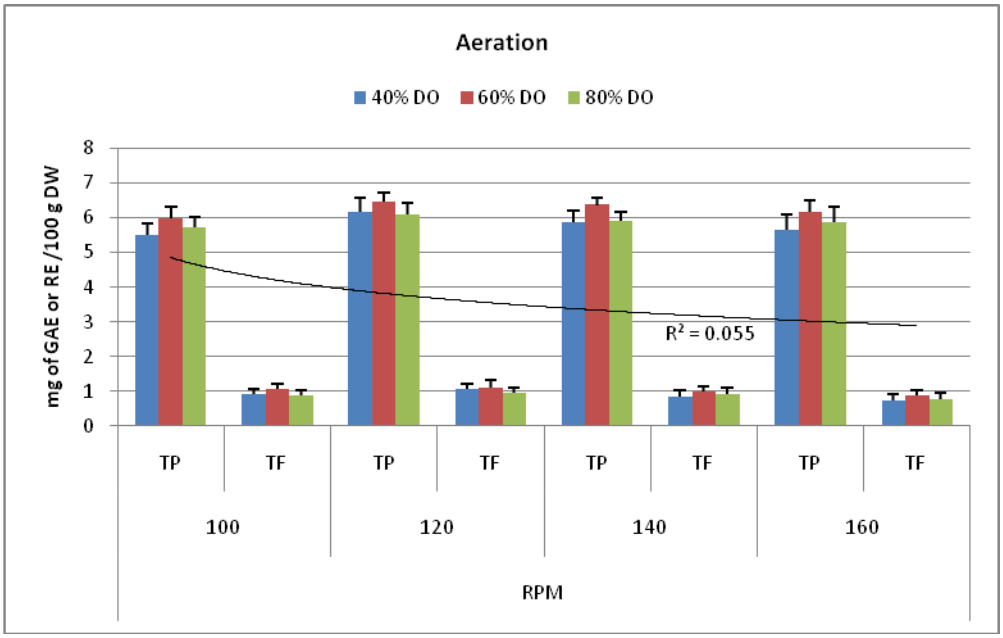

Fig.(3). Effect of different agitation (rpm) and aeration levels DO (%) on TP and TF contents (mg GAE or RE/100 g DW) of Samany date palm cell lines cultured and incubated in bioreactor (STB) at 28 ± 1°C under light condition (2000 Lux) for 2 weeks. Evaluation of antioxidant activity (DPPH assay).

Different methanolic extracts of Samany date palm cell lines affected by different physical parameters (agitation or aeration rates) were tested to investigate their ability to scavenge DPPH on the basis of IC_{50} values. Those IC_{50} values of the methanolic extracts are presented in Table 1. The highest value of IC_{50} (3.13 mg/l) was detected with 100 (rpm) and aeration 40 (%) DO . However the lowest value of IC50 (2.45 mg/l) was detected with 120 (rpm) and 8 (%) of DO which related to the highest antioxidant activity.

Table (1). Antioxidant activity using effectiveness scavenging assay (IC_{50}) on DPPH radical (mg/l) in Samany date palm cell lines cultured and incubated in bioreactor (STB) at 28 ± 1°C under light condition (2000 Lux) for 2 weeks.

Rpm Aeration (DO %)	100	120	140	160
40	3.13±0.376	2.97±0.293	2.95±0.183	2.78±0.187
60	2.89±0.356	2.64±0.225	3.09±0.235	2.96±0.185
80	3.01±0.256	2.45±0.164	2.68±0.197	2.81±0.125

DISCUSSION

Current advances in plant biotechnology provides opportunity to culture plant cells, tissues and organs for the production of useful secondary metabolites instead of whole plant cultivation. **(**Vijaya et al., 2010). Plant cell suspension cultures capable of producing

particular medicinal compounds at a rate similar or superior to that of naturally grown whole plants. However, to fulfill the demand of the increasing population, the major challenge is how to adopt those technologies under laboratory conditions for large scale production of bioactive molecules from plant cells that are reproducible, safe, and economically viable. Further, application of bioreactor technology is the key step toward commercial production of bioactive molecules by plant biotechnology. Compared to naturally grown "whole wild plants" or traditionally grown "whole transgenic plants" their production in bioreactors ensures defined control process conditions. Thus, minimizes or even prevents variations in yield and quality of the products, which simplifies process validation and product registration (Sivakumar, 2006, Eibl and Eibl, 2008). However, it has been claimed that one of the major obstacles in the industrial application of plant cell, tissue or organ culture for the efficient production of commercially important bioactive compound is the low product yield (Zhou and Zhong, 2010). Further, a number of physical and chemical factors that may affect secondary metabolism in plant cell cultures have been explored by Baque et al. (2010) and Chan et al.(2005). Moreover, optimization of process parameters can lead to an enhancement in secondary metabolite production compared intact plants **(**Jeong et al., 2009a; Kim et al., 2005). Bioreactor culture system provides better advantages than the traditional tissue culture system because the culture condition in a bioreactor can be controlled by online monitoring of important process parameters such as temperature, pH,and concentrations of oxygen and carbon dioxide inside the bioreactor vessel. The nutrient concentration can be optimized and nutrient uptake can also be enhanced by continuous medium circulation. Additionally, production cost and time can be reduced by enhancing cell proliferation and regeneration rates, product quality can be controlled, product can be free of pesticide contamination, and product can be harvested all year round to meet the increasing global demand (Paek et al., 2005; Sivakumar et al., 2005, Yansong et al., 2008).

This reporting research may be is the first investigation subjected to the effect of physical conditions agitation and aeration affecting on mass cell production; TPC , TFC and antioxidant activity in Egyptian samnay date palm cultured in STB. Concerning the obtained results with Samany date plam suspension cultures which cultured in STB, it recognized that the optimum conditions to get mass cell fresh or dry weights were recorded with 120 of rpm and 40% of aeration DO. The obtained results were in close with Dornenburg and Knorr (1995) and Smart and Fowler (1981) they reported that plant cells require less oxygen compared to microbial cells, because of their slow metabolism. Further, may be concerning

this case, the air-lift bioreactor is considered as the most desirable reactor for efficient cultivation of suspension cells. Further, the high speed of bladed turbine impellers in STB might be sensitivity sheared of date palm cell cultures. In this respect, Chattopadhyay et al. (2002) and Zhong, (2010) stated that the wall growth and clumping of cells inside the reactor vessel caused by modified rheological nature of the fluid, result in sedimentation that significantly affects cell growth and product formation during bioreactor cultivation.

Moreover, Dae-Sung et al.(2008**)** reported that 43% DO, two of the agitation rate (120 and 225 rpm) resulted in more than 200 % increase in biomass of oil palm suspension culture compared to the initial inoculum. Moreover they reported that excessive agitation (335 rpm) was correlated with poor growth.Regarding of TPC and TFC which recorded 6.45 mg GAE/ 1100 g DW and 1.12 mg RE in suspension cultures of Samany date palm cultured in STB with 120 rpm and aeration 60 % DO**.** Moreover**,** and in close of obtained results Schlatmann et al. (1995) rported that oxygen requirements of plant cells are relatively low for cell growth, but may significantly increase during metabolite synthesis. On other hand Mansouri et al.(2005) reported that TPC of kharak date palm fruits(DPF) varied from 2.89 to 141.35 mg gallic acid equivalents (GAE)/100 g dw sample . However, Al-Farsi et al. (2007) reported TPC values between 172 and 246 mg gallic acid equivalents/100 g fresh weight of Omani dates. Moreover, and in line with recorded results Mansouri et al. (2005) and Al-Farsi et al.(2007) they demonstrated that total flavonoid content (TFC) of DPF measured using aluminum chloride colorimetric methods varied considerably from 1.62 to 81.79 mg in terms of catching equivalents/100 g dw of sample. Concerning the antioxidant activities **t**he best results of antioxidant activities of IC_{50} was 2.45 mg/l obtained using physical condition of 120 rpm and aeration 80% DO. Variable results have been reported on the relation between TPC and antioxidant activity of different natural products. Some authors found a correlation between TPC and the antioxidant activity (Velioglu et al., 1998, Adom and Liu 2002 , Yousfi et al.,2009, Gruz et al.,2011) while others did not (Zielinski and Kozlowska 2000, Maillard and Berset 1995, Hahkonen et al., 1999).

CONCLUSIONS

The present study showed that plant bioreactors are attractive expression systems for economic production of pharmaceuticals. This study need more investigations relation with the type of effective bioreactor (s) , physical and chemical condition affecting on scaling up and semi-industrial production of pharmaceutical compounds.

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