Optimization of Conditions For Increasing of Saffron Cell Biomass And Crocin Production In Stirred Bioreactor

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Abstract

Bioreactors provide suitable conditions for the growth of cells and production of secondary metabolites by regulating physical and chemical factors. In this study, first, sucrose, 2-(N-morpholino) ethanesulfonic acid (MES) as a buffering agent and medium pH was optimized in the Erlenmeyer flask. This aim was then pursued in a stirred bioreactor through aeration and pH medium adjustment. Results of the first step showed that Schenk and Hildebrandt (SH) basal medium with naphthalene acetic acid (2 $mg.l^{-1}$) and 6-benzylaminopurine (1 $mg.l^{-1}$) supplemented with 2.5 mM of MES and gradually increment of sucrose from 3 to 6% caused to catch the highest cell biomass and crocin production. The spectrophotometry measurement showed that the highest crocin content of the cells was 0.8 mg/g after five weeks. The results of the second part revealed that in the stirred bioreactor, constant pH (5.8) during the growth period is a limited factor for the cell growth and crocin production. Although aeration initially found to be an inhibited factor for the production of crocin, results showed that, if the evaporated volume of water caused by aeration is constituted, it can be an effective factor to increase cell growth rate around 2 folds. In addition, total crocin content of the cells, based on the HPLC could be raised up to 2 mg/g. Based on this study, it can be concluded that MES and gradual increment of sucrose could increasing the cell growth and crocin production. Aeration in bioreactor can increase cell biomass, if the medium volume will be kept constant.

Key Message

Saffron, the most expensive spice contains valuable compounds like crocin. Corm derived-cell containing crocin can be produced in higher scales and cheaper price by using cell culture in stirred bioreactor.

1- Introduction

Secondary metabolites are widely used directly or indirectly in the industries such as pharmaceuticals and food industries (Zhao et al. 2005). Although the potential of medicinal plants in the production of secondary metabolites seems limitless, their use in modern pharmacy is limited (Gorelick and Bernstein 2014). Some of the limitation factors are diversity of natural compounds in plant extracts, the complexity of their chemical structure and their dependence on environmental conditions, which has made the standardization of herbal medicines very difficult (Schmidt et al. 2007).

Cell suspension culture technique is a new method for the production of plant secondary compounds under controlled conditions and is considered as promising approach in the industrial production of natural compounds (Karuppusamy 2009). This technology has many advantages including safety, predictable production; effective, fast and cost-effective separation and purification, the ability to use elicitors; simple standardization, metabolites traceability, independent production to the environmental conditions (Rao and Ravishankar 2002; Nalawade and Tsay 2004). Despite these advantages, this technique has associated with some disadvantages. The first and most important challenge is the low accumulation of metabolites in cells grown in *in-vitro* conditions. To solve this challenge, several

solutions including optimizing the medium compositions, acidity, type and concentration of plant growth regulators and carbohydrates, application of elicitors and precursors, regulation of oxygen levels and aeration have been suggested (Kolewe 2011). Researches have shown that optimization of these factors can play an important role in the production and accumulation of secondary metabolites, but this might be varied based on the plant and proposed compound (Rao and Ravishankar 2002; Pitzschke and Hirt 2010).

Two of these factors whose their optimization in cell suspension cultures has a significant effect on cell biomass and production of secondary metabolites are pH and aeration (Kevács et al. 1995). The medium pH plays an important role in the cell growth and production of secondary metabolites due to affecting on rate of solubility of elements as well as their uptake (Malik et al. 2008). pH variation is influenced by several factors, such as initial pH, autoclaving, cell growth rate, and cell culture period (Andersone and levinsh 2008).

The only element which is able to create buffering properties and prevent pH fluctuations in plant *in vitro* culture is phosphate compounds. Regarding the fact that the medium content of this element is very low and with such a low amount of phosphorous, there is no ability to show the buffering properties due to consuming of phosphorous during the early steps of cells growing. Accordingly, the fluctuations in pH will happen in response to the uptake of ammonium and nitrate(Leifert et al. 1992, 1995).

So far, several studies have been conducted to use buffers to prevent pH variation in solid media and their effect on *in vitro* growth factors (Parfitt et al. 1988; Tu et al. 1996; Baker et al. 2007; Andersone and levinsh 2008; Kagenishi et al. 2016; Thorat et al. 2017; Ramulifho et al. 2019), but there is no repot on the use of buffers in plant cell suspension cultures to address their effect on cell biomass growth and metabolites production.

Nowadays bioreactor has become an attractive device in the field of plant cell cultures due to its ability to provide suitable conditions for cell growth and production of metabolites. In a bioreactor, the factors such as oxygen concentration, medium temperature, pH and agitation can be under the control (Paek et al. 2014). Among these factors, aeration and oxygen providing in plants cell suspension cultures has attracted the attention of researches (Ahmed et al. 2008; Dong et al. 2013; Piao et al. 2017). Aeration, in addition to the effective on providing dissolved oxygen, cell activities and biomass; it also improves the accessibility of the cells to nutrients and production of metabolites by mixing the medium. (Ahmed et al. 2008). Although aeration rate has a key role on oxygen transfer, once is not optimal, it will cause shear stress (Meijer et al. 1993). Therefore, one of the most important factors that need to be optimized in the bioreactor is aeration (Piao et al. 2017). It is given that the response of different plants to pH fluctuation or aeration rate varies during the period of suspension cell cultures. It is therefore, the achievement to the most suitable conditions for each plant need to be studied (Chen et al. 2015; Pujol 2016).

Saffron, which is obtained from the stigmas of flowers of *Crocus sativus* L. is the most expensive spice in the world. Despite the proven medicinal properties of saffron, its application in related industries is limited due to its high price; hence its consumption has remained somehow traditional (Soeda et al.

2007). The advantages of cell cultures in saffron can be raised when applied in a bioreactor because of the production of valuable metabolites would be at a lower cost. Despite this advantage, there is no report yet in case of saffron cell culture. In the present study, for the first time, the saffron cell culture was optimized in terms of pH, sucrose concentration in order to increase cell biomass and crocin production in a shacked flask system. In the final part of the study, the effect of constant and variable pH of medium during the period of cell growth, as well as the role of aeration on increasing of cell biomass and production of crocin were investigated in a fed batch system stirred bioreactor

2- Materials And Methods

2-1- Callus Induction and establishment of cell suspension cultures

In this study, saffron corms were used as plant material for callus induction. Results of our previous researches have shown that mature corms compared to immature ones, have the ability to induce more callus and consequently cells with higher amount of metabolite (unpublished). Accordingly the corms used in this study were adult with the size of at least 4 cm in diameter. All corms were harvested from a farm in Mashhad- Iran in middle of May and transferred to the Laboratory at the Research Institute of Food Science and Technology. The steps from callus formation to the establishment of the suspension cell cultures are shown in Figure 1.

After removing the outer shells, the corms were disinfected with 70% EtOH for 1 min and then 1% sodium hypochlorite for 15 min. The step was followed by three times rinsing with distilled water, each time for 5 min. Disinfected corms then divided into small pieces and cultured on B_5 medium supplemented with NAA (2 mgl^{-1}), BA (1 mgl^{-1}) and 3% sucrose. It was then solidified with 0.7% agar after adjusting the pH on 5.8. All the solid cultures were incubated at 23 ± 2 °C in the dark conditions and sub-cultured on the same medium compositions after 4 weeks. After that, to establish the suspension cultures, fresh callus was inoculated into 100 ml flask containing 20 ml liquid SH medium supplemented with NAA (2 $mg.l^{-1}$), BA (1 $mg.l^{-1}$) and 3% sucrose. All the cultured flask cells were placed on a rotary shaker at 110 rpm at 23±2 °C in darkness for 2 weeks to acclimatize with new medium. At the end of this period, suspension cultures were applied with the desired treatments.

2-2- Saffron cell suspension cultures

In this part of the study, the effect of pH medium, sucrose concentration and gradual increment of sucrose on cell mass growth and crocin production was investigated in saffron cell suspension culture. In order to investigate the possibility of pH stabilizing and its effect on cell growth and crocin production, MES (2- (N-morpholino) ethanesulfonic acid) at three concentrations (0, 2.5 and5 mM) was used. The effect of sucrose was investigated at three concentrations of 3, 6 and gradually 6%. In the gradual addition of carbohydrates, the initial concentration of sucrose was considered 3%. After seven days, addition of autoclaved sucrose solution was began on a weekly interval, so that, in the sixth week it reached the final concentration of 6%. To eliminate the error the same amount of sterile distilled water

was added to the control cultures. The experiment was performed in 100 ml Erlenmeyer flasks containing 20 ml of SH culture medium supplemented with NAA (2 $mg.l^{-1}$) and BA (1 $mg.l^{-1}$). One gram of cells obtained from previous cell suspension establishment was inoculated into each flask. The parameters of cell growth index, number of cells in the liquid medium and pH of the culture medium were measured weekly. The amount of crocin in each flask was measured by spectrophotometry at the end of the fourth and sixth weeks.

2-3- Saffron cell culture in stirred bioreactor

In order to investigate the role of pH stability and aeration on saffron cell growth and crocin production, two experiments were planned in a stirred bioreactor, a Hanil Liflus FX bioreactor with two three-liter vessels. In the first experiment, the effect of pH and aeration was studied on saffron cell growth and crocin production. To this purpose, the pH was considered to be constant or non-constant with or without aeration. For both conditions, the initial pH was adjusted on 5.8 , but for the stable one by addition of NaOH (0.5 N) or HCl (0.1 N) , the pH was automatically adjusted on 5.8 during the growth period, while there was no adjusting for the non-constant pH, so the pH varied naturally during the culture period. The aeration was in two levels, with (0.5 VVM [1]) or without aeration. In the second experiment, the effect of aeration was addressed on cell growth index and crocin production. In this part, to avoid from the side effects of water loss due to the aeration and evaporation, the medium volume was kept constant by adding double distilled water into the vessel during the growth period in an interval of 2-3 days.

The medium was prepared similar to the previous experiment. Each vessel containing SH medium (1 L), which was inoculated with 50 g fresh cells (5% w/v). Agitation for both vessels was designed to be on 80 rpm with a pitched blade and kept in dark conditions at 23±2 °C for 5 weeks. At the end of this period, growth index of the collected cells from bioreactors was measured. The crocin content of dried cells was also measured by HPLC method at 440 nm.

3- HPLC analysis

Crocin was identified and quantified by HPLC using a Waters 1525 binary HPLC pump, equipped with Waters 2489 UV/Visible Detector, and Breeze software. Column C18, ODS. 250 mm, 4.6 mm, particle size 5.0 µm was employed. Methanol (HPLC grade) with gradient flow rate (20 to 80%) at 1 ml/min was used as mobile phase. Detection wavelength adjusted on 440 nm for crocin detection. Duration of the test was 60 min and the volume of the injection was 20 µl. Temperature was adjusted on 30 ˚C.

Standard crocin sample was prepared as dissolving 10.0 mg crocin in 5.0 ml of MeOH: water (50:50) to make a concentration of 2000 ppm. For HPLC analysis of crocin, Alam's protocol with a bit modification was followed (Alam et al. 2016). In this protocol, 0.07 g of powdered dried cell was suspended in 5.0 ml of MeOH (80%) and magnetically stirred for 24 h at room temperature under dark conditions. After extracting crocin, the sample was filtered through 0.25 µm pore size filter membrane and applied on HPLC. The crocin contents of cell extract was identified and quantified through comparing the retention time and absorbance on UV spectra with crocin standard. Crocin concentration was calculated based on

the peak area of the cell extract sample with the standard and using calibration curve. Finally, the crocin was expressed in mg/g of cells on a dry mass basis.

4- Statistical analysis

Statistical analysis was designed according to the type of experiment and the data were analyzed using SPSS software version 16. In this study, the effect of MES and sucrose percentage was studied by a repeated measurement design with two factors under a completely randomized design with 4 replications over a period of five weeks. The factors in stirred bioreactor were investigated in a completely randomized design for cell fresh weight and crocin data. Mean comparison was calculated by Duncan test at 95% confidence level.

[1]Volume per Volume Medium

3- Results And Discussion

The results of this study revealed that the pH of the medium was influenced by concentrations of sucrose. Actually, medium containing the 3% sucrose showed the highest pH at the end of the growth period while the lowest pH was observed in medium containing 6% sucrose (Figure 3). On the other hand, in the treatment of gradual sucrose increment, the pH was stand in the middle as compared to the 3 and 6% sucrose.

As shown in Figure 4, the concentration of MES also had a significant effect on the medium pH. In the medium with the highest MES content (5 mM), the lowest pH was observed while, no significant differences in pH were recorded between the medium containing 2.5 mM of MES and the control treatment (no MES).pH variation in medium containing different levels of sucrose during the growth period (6 weeks) has been presented in figure 5. In all media, pH during the first week was sharply decreased from 5.8 to 4.37, 4.47 and 4.6 in the medium enriched with 6%, gradually 6% and 3 %sucrose respectively. During the second week, a slight increase in pH was observed in all three media, although the pH increment in medium with 3% sucrose was not significant, in others it raised up significantly. In the third week, the pH was constant in all three media and no change was observed. In the fourth week, it was experienced a decrease in pH in all three culture media. Although the pH decrement in medium with 3% and 6% (gradual) was significant, but in the medium containing 6% sucrose, the decrement was not significant. In the fifth week, pH in all three treatments was sharply increased. The highest and the lowest pH was observed in the medium with 3% and 6% sucrose. However, it should be noted that at the end of the period, although, no significant difference was observed between the pH in 6% and 6% (gradual) sucrose, the pH in the medium containing 3% sucrose was significantly higher than the others.

The trend of pH changes under the influence of different MES concentrations was similar to those observed when it was influenced by the different levels of sucrose (Figure 6). In fact, in the first week, although a sharp decrement was observed in the pH, there was no significant difference between the MES treated and non-treated one. During the second week, an arising trend was observed in both

concentrations of MES, which are significantly different from the previous week, but in the control, it was non-significant. Indeed, at the end of this period, no significant difference was observed between MES treated and non-treated samples. Although, the trend of pH increment was continued during the third week, it was not significant compared to the previous week. In the fourth week, a decrement in pH was observed in all treatments, so that, it was significant for MES treated and non-treated samples. However, it should be noted that at the end of fourth week, no significant differences was seen among all treatments. Finally, in the fifth week, the pH in all three treatments increased significantly compared to the previous week. Although no significant difference was observed between control (0 mM) and 2.5 mM MES, the medium containing 5 mM MES significantly showed lower pH than others.

As shown in Figure 7, cell growth index had the highest growth during the first week, from 0 to 0.39, while during the second week a dramatically growth was observed, so that, the growth index increased only 0.07. Since that till the end of sixth week, an exponential cell growth was seen and it reached to 1.28.

The interaction of different concentrations of MES and sucrose was found to be statistically significant on saffron cell growth index which has been presented in Figure 8. Results revealed that the highest growth index was happened in the medium contained 2.5 mM MES and 6% sucrose (gradual). Although this treatment does not show significant difference to the control (3% sucrose and no MES), there is a big difference compared to the others treatments. Results showed that in the sucrose content of 6%, though by increment of MES from 0 to 2.5 the cell growth was non-significant, by doubling the concentration of MES (5 mM) the cell growth significantly decreased. Similar trend in cell growth was also observed for 6% sucrose (gradual) except the cell growth significantly raised up with changing MES from 0 to 2.5 mM and then dropped down when the levels of MES increased up to 5 mM. In general, except for 3% sucrose, in the other sucrose contents (6% and 6& (gradual)), MES at 2.5 mM caused the acceleration of cell growth index, but at 5 mM, it had as a limited growth role.

Cell count in the cell suspension cultures showed that the highest number of viable cells was observed in treatment containing 6% sucrose (gradual) supplemented with 2.5 mM MES (Figure 9). In the medium containing 6% sucrose, both with and without MES, the number of cells was significantly reduced. In the medium with 3% sucrose, there was no significant difference with 6% sucrose in terms of cell number, either in the presence (2.5 mM MES) or in the absence of MES. The number of live cells increased up in the medium with 3% sucrose, while it decreased in medium containing 6% sucrose (gradual), when the MES level rose from 2.5 to 5 mM.

The spectrophotometry recorded data at 440 nm, related to the interaction of sucrose concentration and sampling time on crocin percentage are presented in Figure 10. Crocin levels were assessed at the end of the fourth and sixth week after the culture time. As shown in this figure, the cell sampling time has a significant effect on the crocin content of the extract. After the five weeks, the lowest and the highest amount of crocin was observed in the medium containing 3% and 6% sucrose (gradual) with 0.47 and 0.8% respectively ($p \leq 0.05$).

Based on the data recorded at the end of fourth and sixth weeks, the interaction effect between MES and sucrose concentrations on crocin production showed that, the highest amount of crocin was observed in medium containing 6% sucrose (gradual) and 2.5 mM MES after five weeks ($p \leq 0.05$). It was also revealed that in this medium, no significant difference was occurred in crocin content when the MES level increased up to 5 mM (Figure 11).

Investigation on cell biomass and crocin production in a stirred bioreactor

The results of this part of the study showed that the constant pH (adjusting pH by adding HCl and NaOH via peristaltic pumps which were automatically under the control of the pH sensing electrode during the growth period, the pH of the medium was kept constant at 5.8) with or without aeration severely reduced the cell growth (Figure 12).

The highest cell growth was observed when the pH variation was happened naturally during the culture period with no effort to keep it constant. Results also revealed that the constant pH and aeration simultaneously led to a decrease the cell biomass in the bioreactor. Consequently, it was also caused a negative effect on crocin contents of saffron cells extract. As shown in figure 12, although the non-constant pH of medium resulted higher crocin content as compared to the constant pH, it was significantly lower to those observed in medium without aeration. On the other hand it was also found that only the extracted cells from cultures with natural pH (non-constant) contained crocin.

The HPLC profiles of the extraction of cell sample were presented in Fig. 13. It showed that the cell extraction sample contains 2 analogues of crocin. The total amount of crocin in cells based on the HPLC results at 440 nm, and crocin standard curve was found to be 2 mg per gram of cell dry weight.

As the growth period of plant cells in suspension cultures takes several weeks, generally a significant volume of medium is losing due to evaporation particularly by aeration in bioreactor system. Accordingly, it the second experiment of the bioreactor, the effect of aeration and volume of medium was addressed. The results of this part of the experiment showed that adjustment of medium volume by adding water led to increased cell growth. Based on this result, it can be concluded that aeration, itself, is not a reason for reduction cell growth and cell browning as happened in the previous experiment. Therefore, increasing the concentration of elements in the medium caused by evaporation can be the reason for cell browning and reducing cell mass growth. Hence, in the aeration system, if the volume of medium can be kept constant during the culture, aeration can have a positive effect on cell growth. The results of this section are presented in Figure 14.

4- Discussion

The results of this study showed that no buffering effect was observed for MES at tested concentrations (2.5 and 5 mM) in saffron cell suspension culture. Although, there are several reports on buffering role of MES in plant tissue culture or hydroponic, no reports have been published of the use of MES in suspension cell culture so far (Tu et al. 1996; Baker et al. 2007; Kagenishi et al. 2016; Thorat et al. 2017).

Kagenishi et al. (2016) reported that the most appropriate concentration of MES in *in vitro* conditions for rooting is 0.1% (5 mM), while they found that MES at level of 1% (50 mM) is toxic and has an inhibitory effect. In another study, it was reported that MES even at lower concentrations (5 mM) has a toxic effect such as increasing oxidation of some phenolic compounds (Baker et al. 2007) or formation of abnormal somatic embryos at the level of 10 mmol (Tu et al. 1996). On the other hand, Thorat, et al. (2017) reported that MES at applied concentration (500 mg/l) (2.5 mM) had a buffering role in the medium and led to increased callus formation. They believed that MES at this concentration was able to control the pH of the medium (Thorat et al. 2017). According to the above mentioned results, applied concentrations of MES in the present study was in non-toxic range and consequently no toxicity and abnormal growth were observed in the cells. It was also found that, buffering effect of MES is strongly influenced by its concentration. Nicholas and Harper (1993) reported that MES has a buffering role only in a period of 5 days after adding to the culture, while in the present study, the first pH recording was happened after 7 days from the culturing time, means we likely lost the opportunity to record the buffering role of MES. Interestingly, as a new finding in this study, it should be stated that, MES had an increasing effect on the cell biomass and crocin content.

Another investigated factor in this study was the effect of sucrose and its concentration on cell biomass and crocin production. Carbohydrates as an essential component in the medium, is necessary for cell division and differentiation. They also affect the cell growth factors by creating osmotic potential (Sotiropoulos et al. 2006). Scientific reports showed that carbohydrates in the medium have an effect on the expression of many genes and synthesis of related metabolites (Koch 1996). Plants requirements to carbohydrates are strongly influenced by plant species and their growth stage (Thompson and Thorpe 1987). The results of several studies in plant tissue culture in use of sucrose as carbon source revealed that increasing the concentration of sucrose in the medium leads to increased production of phenolic compounds but causes to reduction of growth, means that it is harmful to plants at higher levels (Hilae and Te-chato 2005; Yildiz et al. 2007; Yaseen et al. 2009). These results confirm the findings of this study, in which sucrose at concentration of 6% led to cell browning and decreased growth index. One of the reasons for cell browning damage is mainly attributed to a decrease in osmotic potential and an increase in the stress signal to the cells (Ahmed et al. 2008) which may happen by increasing sucrose at once. The remarkable point of this study is the gradual increase of sucrose in the medium, which not only did not make an inhibitory effect, but also stimulated cell biomass and crocin production. Sucrose feeding was started at the end of the first week, when the first medium pH was recorded. As shown in figure 4, end of the first week is the time that pH dropping is ended and the stabilization period started. It is also the time that the nitrate uptake increasing and due to making a ion balance in the cells, the carbohydrate uptake also arising (Kevács et al. 1995). When the pH is reduced and the cells need more energy, an increasing in sucrose can probably stimulate cell biomass and crocin production.

The results of this study showed that aeration can increase cell biomass, if the medium volume will be kept constant. This means that the evaporated volume of medium caused by aeration should be replaced. Generally, aeration can increase water evaporation, followed by raising the concentration of elements and compounds in the medium, which is toxic for the cell. Accordingly, in this study, the

equivalent of the lost volume of medium caused by evaporation was compensated with sterile water to keep the volume constant. There are several reports on the effect of aeration on cell biomass and production of metabolites in bioreactors. In most of them, aeration has been introduced as a suitable factor for plant cell growth (Jeong and Park 2005; Dong et al. 2013) that confirm the obtained results of this study. In a review of several reported results, it can be stated that although decreased aeration can limit cell growth, the increased aeration is also caused shear damage and browning the cells (Rao and Ravishankar 2002) Therefore, the amount of aeration for each plant is a certain threshold. Dong et al (2013) showed that the aeration in the range of 0.1 to 0.4 VVM increases, while over 0.5 VVM, decrease the cell biomass. The results of present study showed that, although aeration increased the cell biomass, it apparently has a limitation factor on formation of crocin in tested conditions. So far, no clear relationship has been pointed out between the amount of aeration and the production of secondary metabolites. Dong et al. (2013) reported that increased aeration in ginseng suspended cell cultures can reduce the amount of secondary metabolites while results of Min et al. (2007) showed that there is a direct relationship between aeration and tropane alkaloid production in *Scopolia parviflora* adventitious root culture. In contrast to the above mentioned results, Wang and Qi (2010) reported that raising the aeration in a bioreactor neither increase nor decreases the production of triterpenoids and flavonoids in the cells of *Glycyrrhiza uralensis.* It is therefore, the optimization of aeration in bioreactor is strongly influenced by plant species.

Abbreviations

BA: 6-benzylaminopurine MES: 2-(N-morpholino) ethanesulfonic acid NAA: naphthalene acetic acid SH: Schenk and Hildebrandt (1972)

Declarations

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Availability of data and material: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Code availability: Not applicable

Author contributions: In this experiment, Ziaratnia and Hemmati designed the experiments. Amini carried out all the experiments, analyzed data and wrote the paper. All authors read and approved the manuscript.

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Figures

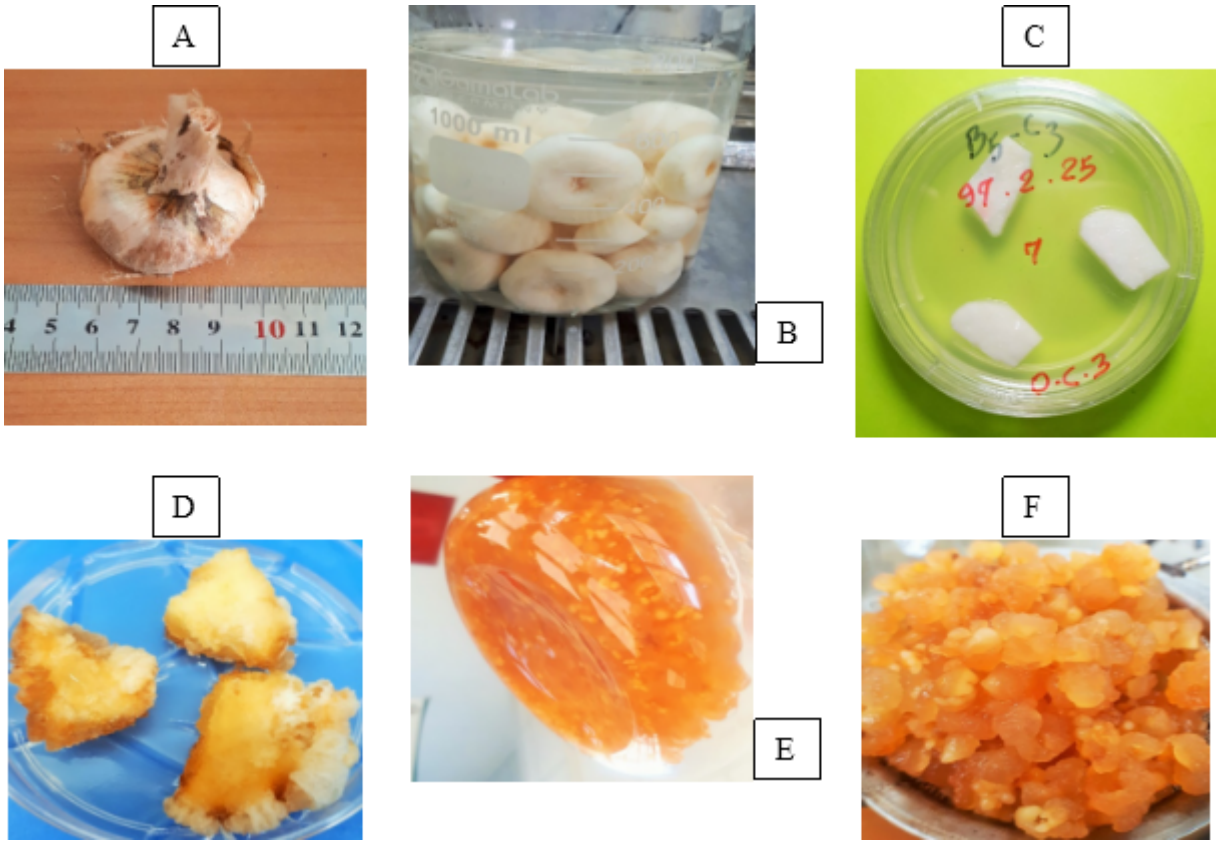

Figure 1

Saffron cell suspension cultures, steps from corms to the induction of corm-derived callus and establishment of cell suspension cultures. A; selected mature corms in May, B; Remove outer shells and disinfection of corms, C; In vitro cultures of explants on solid medium, D; Explant callogenesis, E; Establishment of the cell suspension , F; Formation of aggregated cell in liquid medium

Figure 2

Saffron cell culture in a stirred bioreactor

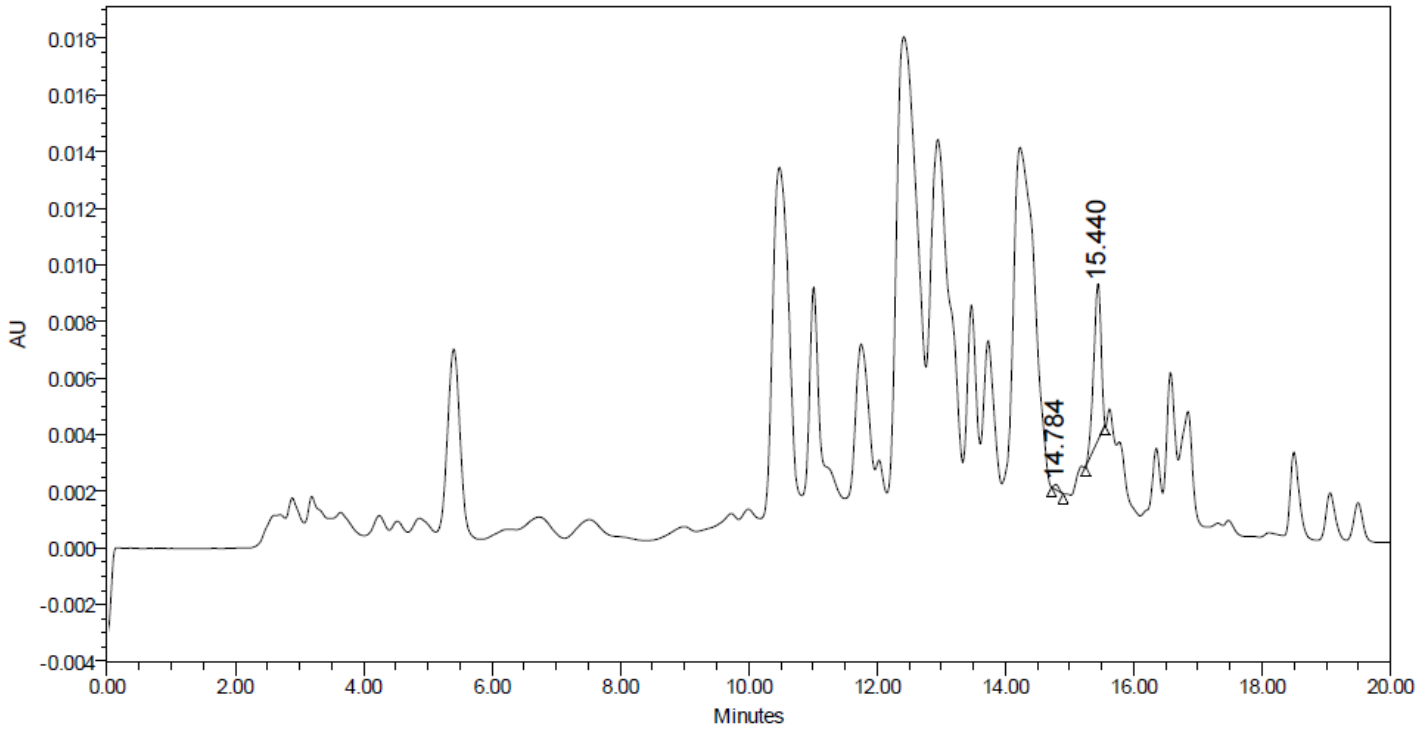

Figure 3

Effect of different concentration of sucrose on medium pH (p≤0.05)

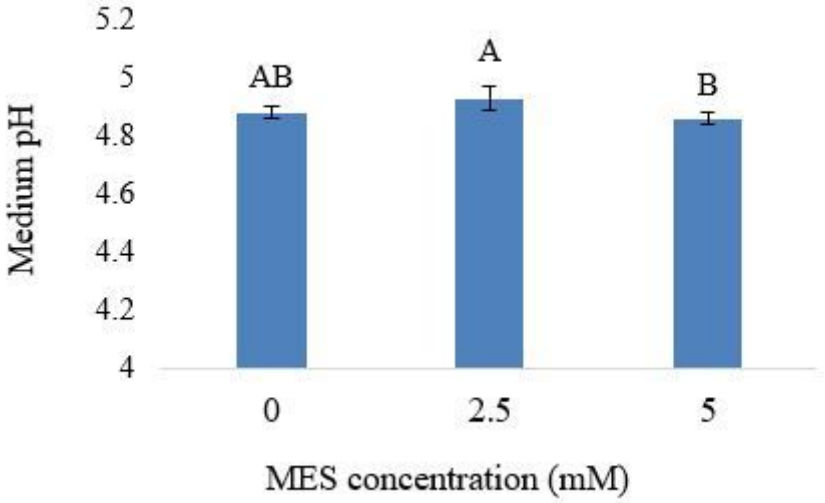

Figure 4

Effect of MES concentrations on medium pH (p≤0.05)

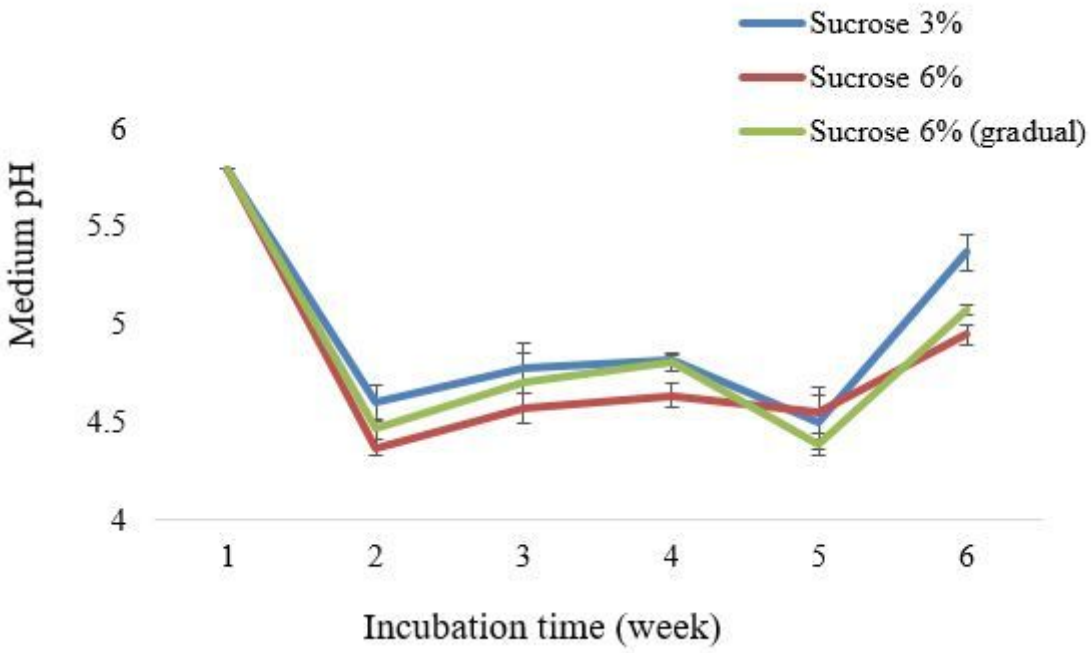

Figure 5

Effect of different concentrations of sucrose on pH of the medium during 6 weeks (p≤0.05)

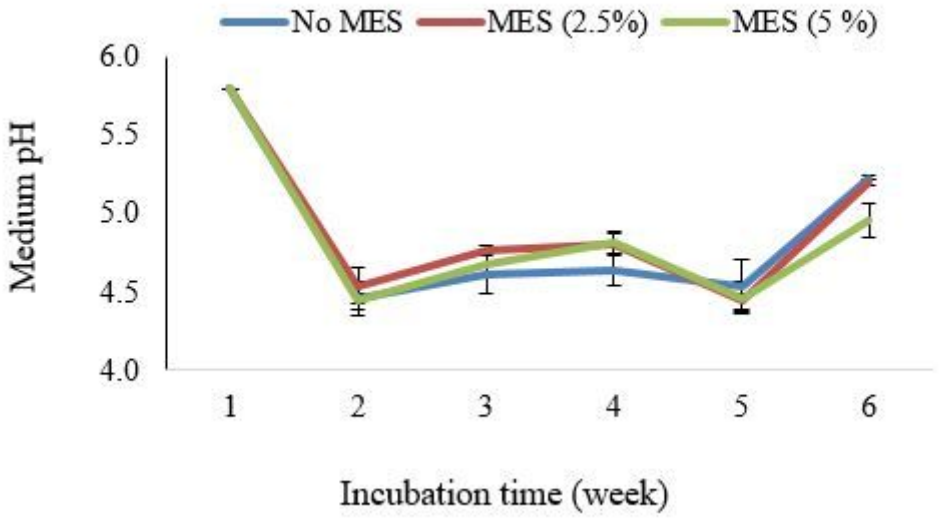

Figure 6

Effect of different concentrations of MES on pH of the medium during 6 weeks (p≤0.05)

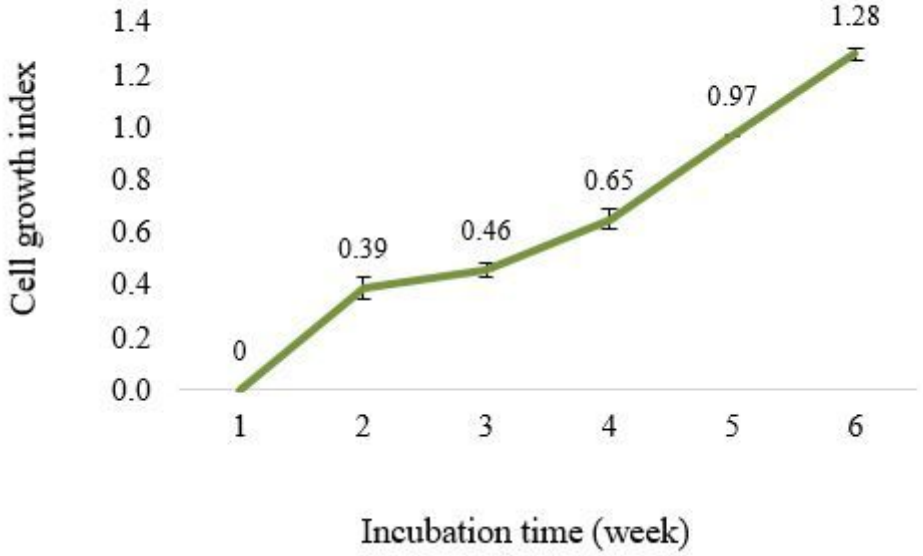

Figure 7

The cell growth index over six weeks (p≤0.05)

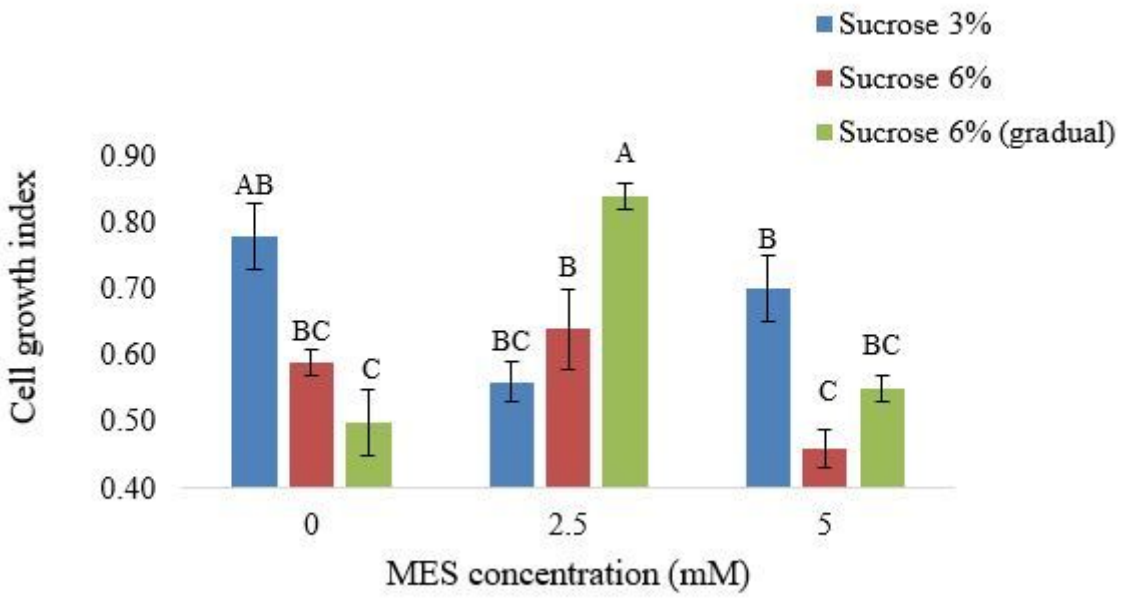

Figure 8

The interaction effect of different concentrations of MES and sucrose on saffron cell growth index ($p \leq 0.05$)

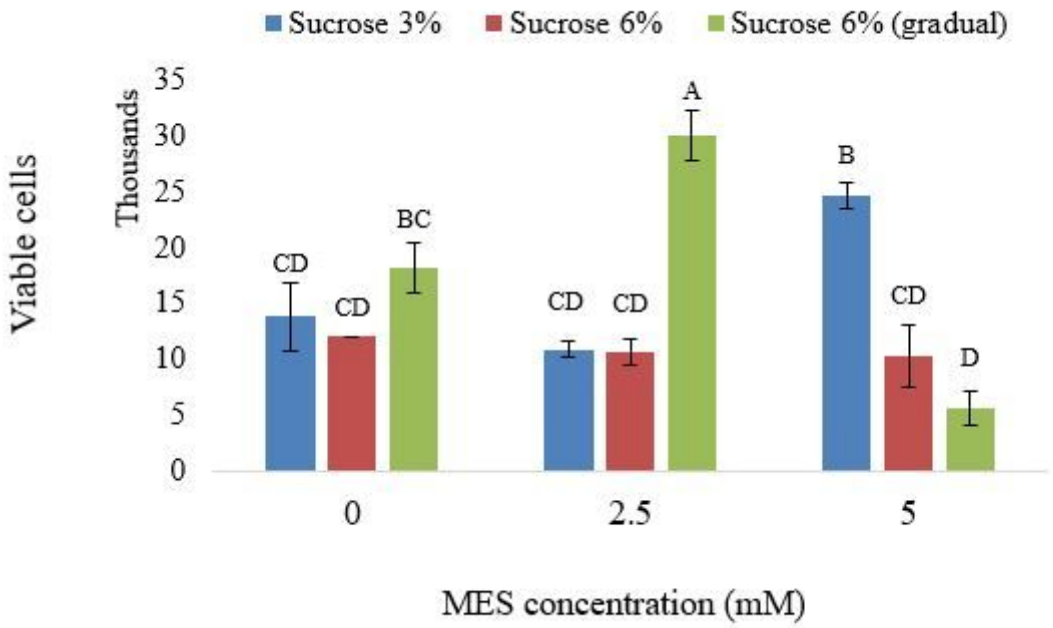

Figure 9

The interaction effect of different concentrations of MES and sucrose on saffron cells number ($p \leq 0.05$)

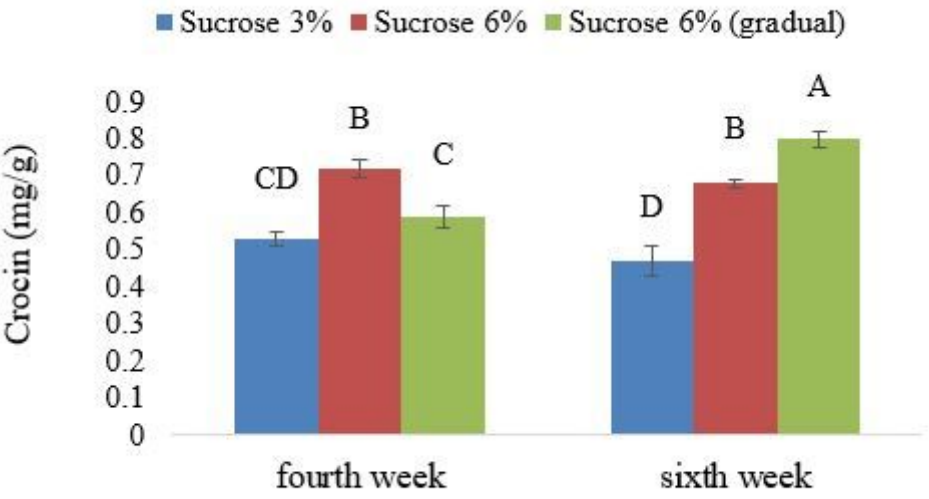

Figure 10

The interaction effects of different concentrations of sucrose and cell sampling time on the crocin content of saffron cell extracts ($p \leq 0.05$)

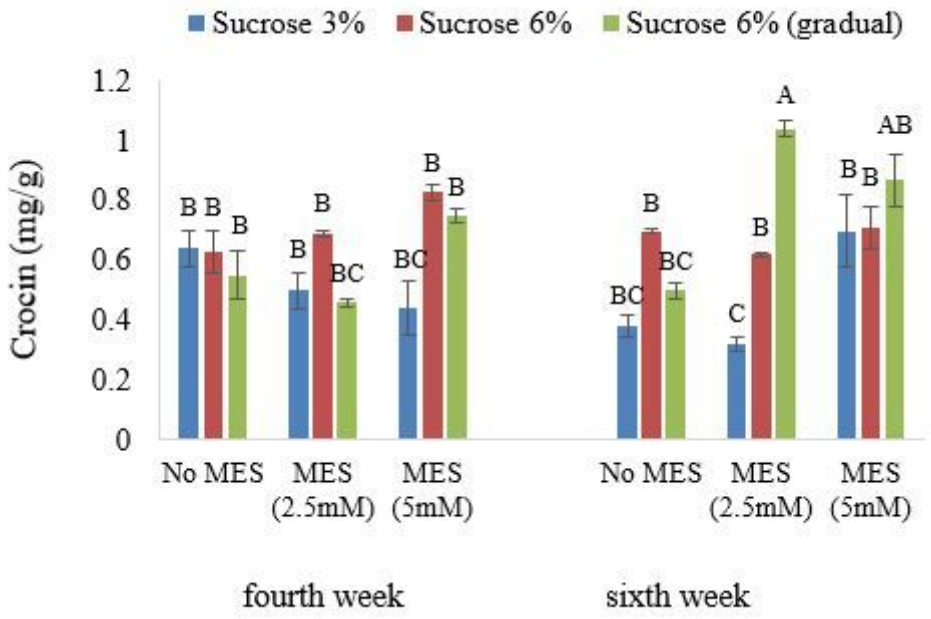

Figure 11

The interaction effect of different concentrations of MES, sucrose and cell sampling time on crocin contents of saffron cell extracts ($p \leq 0.05$)

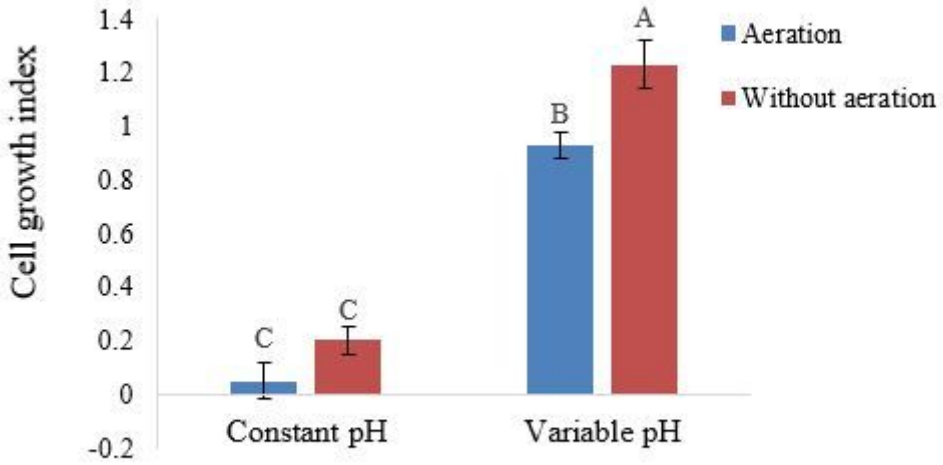

Figure 12

The interaction effects of aeration and pH conditions on crocin contents of saffron cell extracts collected from stirred bioreactor ($p \leq 0.05$)

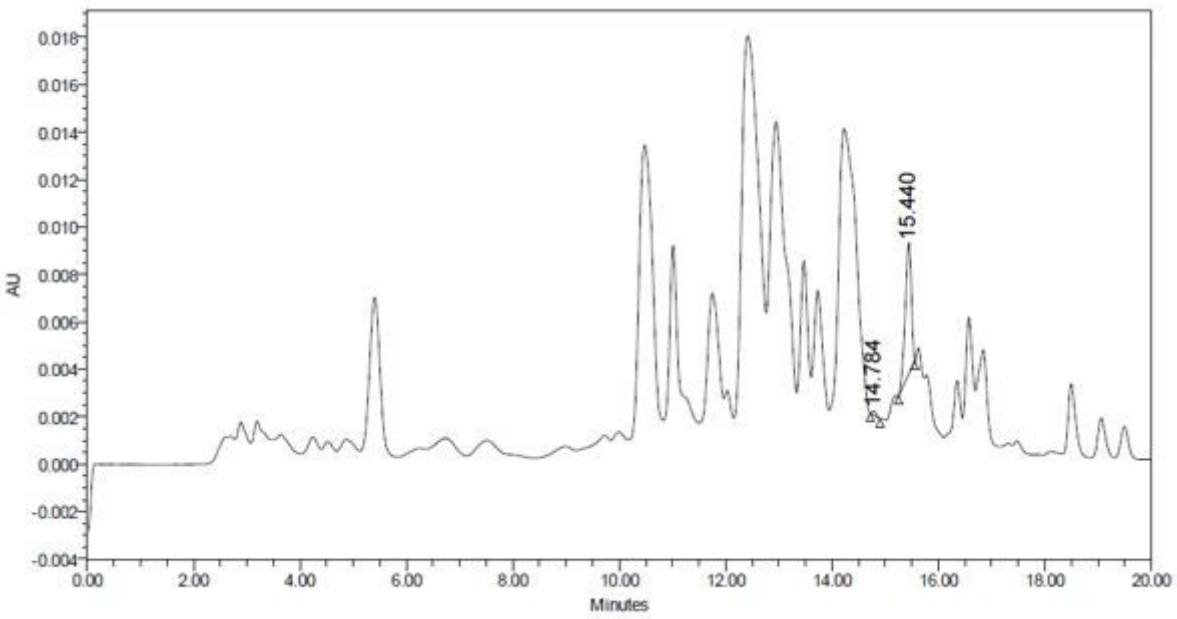

Figure 13

HPLC chromatogram for detection of crocin content in saffron cells extract that grown in bioreactor

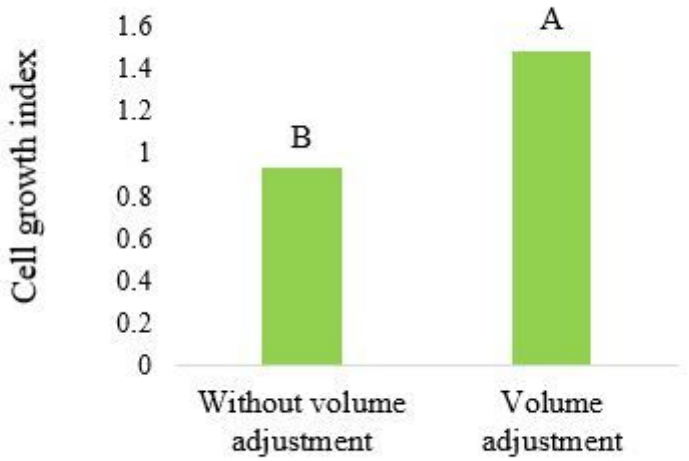

Figure 14

Effect of volume adjustment of medium in bioreactor on cell growth index