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, saffron cell growth and the production of crocin metabolite were evaluated in Erlenmeyer flask and in a stirred bioreactor. To optimize the conditions, the effect of different concentrations of 2-(N-morpholino)ethanesulfonic acid (MES) (0, 2.5 and 5 mM) as a buffering agent and sucrose (3%, 6% and gradually 6%) were addressed on saffron cell growth and crocin production in the Erlenmeyer flask. This aim was then pursued in a stirred bioreactor through investigation on the effect of aeration and pH medium adjustment. The results of the first step experiment showed that Schenk and Hildebrandt basal medium with naphthalene acetic acid (2 mg l−1) and 6-benzylaminopurine (1 mg l−1) supplemented with 2.5 mM of MES as well as gradual increment of sucrose from 3 to 6% caused the highest cell biomass and crocin production. The spectrophotometry measurement showed that the highest crocin content of the cells was 0.8 mg g−1 after 6 weeks. The results of the second part revealed that in the stirred bioreactor, constant pH (5.8) during the growth period is a limiting factor for the cell growth and crocin production. Aeration was also found to be an inhibiting factor for the production of crocin. The results showed that, if the evaporated volume of water caused by aeration is constituted, it can be an effective factor to increase cell growth around 2 folds. In addition, total crocin content of the proliferated cells in stirred bioreactor system under the non-constant pH and without aeration conditions, could be raised up to 2 mg g−1 of cell dry weight, based on the HPLC determination. According to the results of this study, it can be concluded that MES and gradual increment of sucrose could increase the cell growth and crocin production. In the tested stirred bioreactor, it was found that the natural pH fluctuation is a suitable condition for saffron biomass cell growth and crocin production. The proper aeration could increase the cell biomass, if the evaporated water is replaced. However based on the obtained results it can be concluded that, aeration at tested value is not capable of increasing the crocin production.

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.

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**Abbreviations**

BA 6-Benzylaminopurine  
MES 2-(N-morpholino) ethanesulfonic acid  
NAA Naphthalene acetic acid  
SH Schenk and Hildebrandt (1972)

**Introduction**

Secondary metabolites are widely used directly or indirectly in 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 associated with some limitations. (Gorelick and Bernstein 2014). Some of these limiting factors including 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 is a technique for the production of plant secondary compounds under controlled conditions and it is considered as a 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 and independent production to the environmental conditions (Rao and Ravishankar 2002; Nalawade and Tsay 2004). Despite these advantages, this technique has been associated with some disadvantages. The first and the most important challenge is the low accumulation of metabolites in cells grown in *in vitro* conditions. To solve this problem, 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 rate have been suggested (Kolewe 2011). It is believed that optimization of these factors can play an important role in the increment of secondary metabolites in plants cell culture, but depends on the type of plant species and proposed compound, an specific optimization needs to be addressed (Rao and Ravishankar 2002; Pitzschke and Hirt 2010). Two factors whose optimization in cell suspension cultures can have a significant effect on cell biomass growth and production of secondary metabolites are culture medium pH and aeration rate (Kevács et al. 1995).

Intracellular pH is a fundamental physiological factor with a great effect on the growth and metabolism of plant cells due to its wide consequences on the transport of nutrients and hormones enzymatic reactions in the cells. Therefore, cells spend a lot of energy to the regulate cytoplasmic pH (Minocha 1987). Cytoplasmic pH is dependent on the medium pH. Medium pH can have effect on cell permeability and release of secondary metabolites (Mukundan et al. 1998). It also facilitates or inhibits the nutrient availability in the medium (Chen et al. 2014). Accordingly, adjustment of medium pH plays an important role in the cell growth and production of secondary metabolites (De Klerk et al. 2008; Malik et al. 2008).

In plants culture media, in addition to EDTA, amino acids and organic acids with buffering function (Steingroewer et al. 2017), there are alternative mineral elements such as phosphate compounds which also prevent pH fluctuations in the media. Regarding the fact that the buffering contents of plant media are at very low levels, and on the other hand, some of these chemicals like phosphorous compounds were being consumed during the early steps of cells growth, hence they are not able to show an effective buffering properties (Andersone and Ievinsh 2008). Accordingly, plants media are poorly buffered and consequently pH fluctuations were observed during autoclaving and culture period (Andersone and Ievinsh 2008). pH changing generally occurs in response to the uptake of ammonium and nitrate from the medium (Leifert et al. 1992, 1995). Medium pH fluctuations can influence on the yield of biomass and synthesis of metabolites or their release into the medium culture (Banthorpe and Brown 1990; Liu et al. 2002). Although, initial pH of the medium in the most plant species is adjusted to a range of 5.0–6.0 (Loyola-Vargas and Ochoa-Alejo 2018), plants reaction to the fluctuations of pH varies according to specific requirements of individual species (Chen et al. 2014).

MES, has broadly been used as buffering agent in the media (Kagenishi et al. 2016). This buffer is useful to keep the constant pH in the range of 5.5–6.7 and is commonly used for bacteria, yeast, and mammalian cell cultures. Although it is toxic to the plants cells at higher concentrations, it can be used as a buffer agent in plant culture media, at lower concentrations (Pasqua et al. 2002). To date, several studies have been conducted to use buffers with the purpose of prevention of the pH fluctuation and their effect on in vitro growth factors in solid media (Parfitt et al. 1988; Tu et al. 1996; Baker et al. 2007; Andersone and Ievinsh 2008; Kagenishi et al. 2016; Thorat et al. 2017; Ramulifho et al. 2019), but there are few reports on its application as buffering agents in plant cell suspension cultures. For instance, Banthorpe and Brown (1990) have shown that application of MES caused the acceptable cell growth rate and metabolites production in plant cell suspension cultures (Banthorpe and Brown 1990).

Nowadays, cell culture is promising method for commercial production of natural compounds through plant
Bioreactor has become a useful device for scaling up the production of natural compounds due to its ability to provide suitable conditions for cell growth and production of metabolites. In a bioreactor, the factors such as medium pH, temperature, agitation, feeding, carbon dioxide and oxygen concentration can be under the control (Paek et al. 2014). Aeration, in addition to the effect on providing dissolved oxygen, cellular activities and biomass growth; it also improves the accessibility of the cells to the nutrients by mixing the medium. (Ahmed et al. 2008). Accordingly, medium aeration and oxygen supplemented in bioreactor systems has attracted the attention of researchers (Ahmed et al. 2008; Dong et al. 2013; Piao et al. 2017). Despite the effective role of aeration on oxygen transfer, it can lead to shear stress to cells, if not optimized. Shear stress happens when the bubbles ruptured at the surface of liquid (Meijer et al. 1993). Therefore the optimization of aeration rate in a bioreactor is a crucial point (Piao et al. 2017).

One of the most conventional bioreactors is the stirred-tank bioreactor. This bioreactor offers several advantages for culturing plant cells. For example, cell aggregates may be smaller in stirred bioreactors compared to the others types of the bioreactors (Guieysse et al. 2011). Also, stirred vessels are more suitable for viscous broths because of greater power caused by mechanical agitation. The tendency of plant cell cultures to develop dense foam layers at higher aeration rates is an issue that can be eliminate the application of stirred bioreactor. Agitation in stirred bioreactors can be caused by mechanical shear stress. Of course to avoid mechanical damage of the cells associated with high levels of hydrodynamic shear, modified equipment’s and/or operating practices can be used (Doran 2010).

Regarding the response of different plants to pH fluctuations or aeration rate varies during the period of suspension cell cultures, it is, therefore, the achievement of the most suitable conditions need to be addressed for each plant species (Chen et al. 2014; Pujol 2016).

Saffron, a spice derived from the stigma of *Crocus sativus* L., is the most expensive spice in the world. Despite proven medicinal properties of saffron, its application in medicinal industries is limited due to its higher price; hence, its consumption has somehow remained as the traditional healing (Soeda et al. 2007). The saffron cell cultures is economical when it can be scaled up by application of a bioreactor system because of the ability to produce higher amount and lower cost of metabolites. Despite this advantage, there is no report yet in application of bioreactor in saffron cell culture. In the present study, for the first time, the saffron cell culture was optimized in terms of pH and sucrose concentrations in order to increase cell biomass and crocin production in a shacked flask system. In the second 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 cell biomass and crocin production were investigated in a stirred bioreactor.

**Materials and methods**

### Callus induction and establishment of cell suspension cultures

In this study, mature corms of saffron were used as plant material for callus induction. According to the author’s previous study (unpublished) it was found that mature corms in comparison with the immature ones, have higher ability to induce more callus, fine cells and crocin metabolite. The corms used in this study were adult with the size of around at least 4 cm in diameter. All corms were harvested from an experimental farm in Mashhad, Iran in the middle of May and then transferred to the laboratory at the Research Institute of Food Science and Technology. The steps from the callus formation to the establishment of the suspension cell cultures are shown in Fig. 1.

To prepare the explant, after removing outer shells, corms were disinfected with 70% EtOH for 1 min and then 1% sodium hypochlorite solution for 15 min. This step was followed by three times rinsing with sterilized distilled water, each time for 5 min. B. medium supplemented with NAA (2 mg l−1), BA (1 mg l−1) and 3% sucrose. It was then solidified with 0.7% agar after adjusting the pH on 5.8. Disinfected corms divided into small pieces and cultured on solid medium. All the solid cultures were incubated at 22 ± 0.3 °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 flasks were placed on a rotary shaker at 110 rpm at 22 ±0.3 °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.

### 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) ethane sulfonic acid) at three concentrations (0, 2.5 and 5 mM) was used. The effect of sucrose was addressed 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, the addition...
of autoclaved sucrose solution 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.

**Cell growth index measurement**

Cell growth index was calculated based on the following equation (Eq. 1), where $W_0$ is the initial weight of cells at the beginning and $W_1$ is the final weight at the end of the culture period. In this part the fresh cell weight was aseptically measured at the beginning (inoculation) and the end of culture period after filtrating the cells from culture medium.

$$\text{Cell growth index (GI)} = \frac{W_1 - W_0}{W_0}$$  (1)

**Cells counting and cell viability test**

For cells counting and their viability test, first a cell sample was diluted in Trypan Blue dye (0.4%) in a rate of 1:1. A diluted cell sample was added on the hemocytometer chamber and incubated for 1–2 min at room temperature. Finally, the number of cells were counted under the microscope in each square ($1 \times 1 \times 0.1$ mm) of chamber. The total number of cells including non-viable (blue) and viable cells (unstained) based on the Eq. 2 were counted (Singh and Bramhe 2017).

$$\text{Total cells/ml} = \text{total cells counted} \times \text{dilution factor} \times 10,000$$  (2)

In this experiment dilution factor was equal to 2.
Cell extraction

For cell extraction, 0.07 g of saffron dried cell was grounded using mortar and pestle, then 5 ml of 50% ethanol was added for extraction. The mixture was then placed on a shaker with 120 rpm for 24 h at room temperature and dark conditions. To participate the solid particles, the solution was centrifuged at 8000 rpm for 10 min. Finally, the supernatant was transferred into the new micro tube and stored at 4 ºC in the dark for HPLC or UV–Vis analyzing.

UV–Vis spectroscopy

An UV–Vis spectrophotometer (CT-5700, Taiwan), single beam, spectral bandwidth of 2 nm and wavelength range of 190 to 1100 nm, equipped with quartz cuvette (1 cm) was used to determine the crocin contents of saffron cells. 50% ethanol was set as the blank solution. Absorbance of extract was recorded at 440 nm in UV–Vis spectrophotometer. To draw a calibration curve, first standard crocin (C_{44}H_{64}O_{24} Mr 976.98) solution was prepared in the range of 25 to 200 mg l^{-1}, (including 25, 50, 75, 100, 125, 150, 175, 200 mg l^{-1}). The absorption of different concentrations of crocin standard solution was then recorded at 440 nm using UV–Vis spectrophotometer and plotted. Accordingly, Eq. 3 was determined for calculation of crocin contents of the cell extracts (mg l^{-1}). Finally depending on the cell extracts absorption at 440 nm, dry weight of the cells, dilution coefficient and the volume of solvent, crocin content reported in mg per g of cell dry weight.

\[ Y = 0.0128X + 0.0347 \]  (3)

Y is the absorption of cell extract, X is the crocin content (mg l^{-1}).

Saffron cell cultures in a 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 stirred bioreactor, a Hanil Liflus FX bioreactor with two vessels (3 l) (Fig. 2). In this type of bioreactor the medium pH was adjustable with two peristatic pumps (Base and Acid) which were programmable based on time and quantity of adding agents into the vessel. Moreover, the volume of aeration was under the control through a pressure barometer. 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). In the first experiment, the effect of pH and aeration was studied on saffron cell growth and crocin production. Intending to reach this purpose, the pH was considered to be constant or non-constant with or without aeration. For both conditions, the initial pH was adjusted at 5.8, but for the stable one by addition of NaOH (0.4 N) or HCl (0.2 N), the pH was automatically adjusted at 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 (0.5vvm) or without aeration during a culture period. In the second experiment, to avoid 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. Agitation for both experiments was designed to be on 80 rpm with a pitched blade and kept in dark conditions at 22 ± 0.3 ºC for 6 weeks. At the end of the period, growth index of the collected cells from bioreactors was measured. The crocin contents of dried cells was also measured by HPLC method at 440 nm.

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^{-1} 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 at 30 ºC.

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 little 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

1 Volume per Volume Medium.
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⁻¹ of cells on a dry mass basis.

In this research, all chemical compounds were supplied from Sigma Chemical Co, USA.

**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 6 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.

**Results**

**Investigation on the cell growth index and crocin production in Erlenmeyer system**

The results of this part of the study revealed that pH of the medium is related to the concentration of sucrose. Actually, medium containing 3% of sucrose showed the highest pH (4.98) at the end of the growth period while the lowest pH (4.81) was observed in medium containing 6% sucrose (Fig. 3). On the other hand, in the treatment of gradual increment of sucrose, the pH stood in the middle as compared to the 3 and 6% sucrose.

Figure 4 shows the significant effect of different concentrations of MES on pH of the medium (p ≤ 0.5). In the medium with the highest MES (5 mM), the lowest pH (pH = 4.86) was observed while, no significant differences in pH were recorded between the medium containing 2.5 mM of MES (pH = 4.93) and the control (pH = 4.88). Recorded pH in the medium containing different levels of sucrose during the growth period (6 weeks) has been presented in Fig. 5. In all media, pH during the first week sharply decreased from 5.8 to 4.37, 4.47 and 4.6 in medium enriched with 6%, gradually 6% and 3%sucrose respectively. During the second to the fourth week, a slight increase in pH was observed in all three media, although pH increment in medium with 3% sucrose was not significant, in others it rose significantly. During the fourth week, it experienced a decrease in pH in all three culture media. Although pH decrement in the medium with 3% and 6% (gradual) was significant, in the medium containing 6% sucrose, it was not significant. In the fifth week, pH in all three treatments sharply increased.
The highest and the lowest pH were observed in the medium with 3% and 6% sucrose. However, it should be noted that medium containing 6% sucrose was able to have minimum pH changes during the growth (from the second to the end of growth period).

The trend of pH changes under the influence of different MES concentrations was similar to those influenced by the different levels of sucrose (Fig. 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 to the fourth week, although a slight arising trend of pH was observed in all concentrations of MES, which are not significant, the minimum pH changes was observed in the control. However, we experienced a decrease in pH during the fourth week, but 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 Fig. 7, cell growth index had the highest growth during the first week, from 0 to 0.39, while during the second week a slight growth was observed so that the growth index increased only 0.07. Then, an exponential cell growth was seen till the end of sixth week, 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 Fig. 8. The results revealed that the highest growth index happened in the medium containing 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. The 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 rose 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 showed 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 (Fig. 9). In the medium containing 6% sucrose, both with and without MES, the number of cells 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 in the medium with 3% sucrose when the MES level rose from 2.5 to 5 mM, while it decreased in medium containing 6% sucrose (gradual).

The interaction of sucrose concentration and sampling time on crocin content of the cells are presented in Fig. 10. Crocin levels were assessed at the end of the fourth and sixth
week after the culture time. As shown, the cell sampling time has a significant effect on the crocin content of the cell extract. After 6 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 mg g⁻¹ respectively (p ≤ 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 6 weeks (p ≤ 0.05). It was also revealed that in this medium, no significant difference occurred in crocin content when the MES level increased up to 5 mM (Fig. 11).

**Investigation on the cell biomass and crocin production in a stirred bioreactor**

The results of this part of the study showed that the constant pH (pH adjusted on 5.8 during the culture period) with or without aeration severely reducing the cell growth index (Fig. 12). As shown in Fig. 12, not only the constant pH, but also aeration had a negative effect on the cell growth. Therefore, the most suitable condition for the growth of saffron cell biomass in the tested stirred bioreactor is without aeration and pH adjustment. It was also found that, both of aeration and constant medium pH caused a negative effect on production of crocin contents of the cells proliferated in suspension cultures.

The HPLC profile of cell extraction (proliferated cells in non-constant pH and without aeration) is presented in Fig. 13. This figure shows that the cell extract 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 g⁻¹ of cell dry weight. As the growth period of plant cells in suspension cultures takes several weeks, generally a significant volume of medium was lost due to evaporation particularly by aeration in bioreactor system. Accordingly, in the second experiment of the bioreactor, the effect of aeration and volume of medium was addressed. The obtained results 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 main reason for cell browning and reducing cell mass growth. Hence, in the aeration system, if the volume of medium could be kept constant during the culture, aeration would have a positive effect on cell growth. The results of this section are presented in Fig. 14. Interestingly, although aeration with replacement of evaporated volume could recover the reduced cell growth, it could not stimulate the crocin production in the proliferated cells. Therefore, we conclude that aeration in tested conditions in this study (constant or non-constant pH, replacement the lost volume caused by evaporation) had an inhibitor or destruction role on formation of crocin metabolite in the applied stirred bioreactor in this study.

Discussion

The concentration of hydrogen ion (pH) in culture medium is one of the factors that influences accumulation of cellular biomass and metabolites in plant cell suspension culture. It is also reported that the concentration of this ion changes during the culture period (Murthy et al. 2014). PH variations in culture medium occur due to the uptake and utilization of electrolytes from the medium that reflects various phases of the culture cycle. Usually, the pH profile in plant cell cultures shows an initial decrease during the lag phase and a sharp increment during the exponential phase which is followed by a fairly constant value in the stationary phase (Ryu et al. 1990). Accordingly, in the early part of logarithmic phase, the culture medium is more acidic compared to the initial and the stationary phase. This might be due to the production of protons from metabolic activities and rapid removal of nutrient ions from the medium by buffering compounds such as phosphate, EDTA, amino acids or organic acids (Steingroewer et al. 2017). The observed pattern of pH changes in both buffered and un-buffered media in the present study was found to be consistent with the above mentioned results. 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 (Tu et al. 1996; Baker et al. 2007; Kagenishi et al. 2016; Thorat et al. 2017), no reports have been published of the use of MES in the cell suspension culture so far. 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⁻¹) (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. According to the above mentioned reports, although the applied concentrations of MES in this study were non-toxic or no abnormal growth were observed, no significant effect on prevention of pH fluctuations was also recorded. 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. Also, Nicholas and Harper (1993) reported 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 happened after 7 days from the culturing time, it means that we the opportunity of recording the buffering role of MES disappeared. Although in this study we could not see the buffering effect of MES from the end of the first week onwards, according to Nicholas and Harper (1993) results, it can be mentioned that increment of the biomass and crocin was probably related to the inhibiting pH fluctuations in the first few days after the incubation time, when the MES had the more opportunity to play buffering role in the medium.

Another investigated factor in this study was the effect of sucrose and its concentration on cell biomass and crocin production. Carbohydrate 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 have shown that carbohydrate in the medium has an effect on the expression of many genes and synthesis of related metabolites (Koch 1996). Plants requirement to the carbohydrate are strongly influenced by plant species and their growth stage (Thompson and Thorpe 1987). The results of several studies in plants tissue culture, using sucrose as carbon source, revealed that increasing the concentration of sucrose in the medium leads to an increase in the production of phenolic compounds but caused the reduction of growth, means that it was harmful to the plants at higher concentrations (Hilae and Te-chato 2005; Yıldız 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 decrease growth index. One of the reasons for cell browning damage is mainly attributed to the increase of osmotic pressure, and consequently 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 started at the end of the first week, when the first medium pH was recorded. As shown in Fig. 4, at the end of the first week is the time that pH dropping 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 rises (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.

In this study, the cell growth reduction and increment of the cell blackening was found to be the result of the constant pH in the medium in the stirred bioreactor. Generally, in stirred bioreactor, pH adjustment to a certain level needs to be repeated by adding HCl and NaOH. In some cases it required a considerable amount of these chemicals in a long culturing time. It is believed that several adjustment of pH by adding these compounds resulted in increment of Na⁺ and Cl⁻ and decrement of Mg²⁺ and Ca²⁺ in the medium due to precipitation (Shi et al. 2017). In this study, around 40 ml of NaOH (0.4 N) and 25 ml of HCL (0.2 N) was consumed to keep the pH constant on 5.8 in 1 l medium for a period of 6 weeks. Consequently, the addition of these chemicals to the medium for the purpose of pH adjustment may have led to reduction cell growth and then cells death. On the other hand, although pH fluctuation in the medium can be a method to influence the permeability of cell membranes to release secondary metabolites into the medium, if the duration of pH changes is not adjusted accurately, it will lead to cell membrane destruction (Liu et al. 2002). Although there are several reports on the positive effect of artificial changes of pH in medium on extraction of secondary metabolites from cells, hairy roots, cell biomass and on metabolites production (Mukundan et al. 1998; Nagella and Murthy 2010; Paek et al. 2014), there is no report on the effect of constant pH on these traits. In this research it is proposed that a constant pH for such a long time of 6 weeks caused damage to the cells, browning and death.

Hence, improper aeration, not only leads to shear damage in the cells, but also it reduces the volume of the culture medium and then changes some essential compounds concentration which can cause toxicity to cells. Improper aeration sometimes leads to the production of foam on the surface of the culture medium and increases the need to use anti-froam compounds. Therefore, the aeration rate in the bioreactor system requires to be optimized (Ahmed et al. 2008). In this study, aeration was led to a decrease in the level of the medium by increment of evaporation and then decreasing cell growth as compared to culture without aeration. However, in the continuation of the research, the effect of aeration was improved by replacement of evaporated water which then caused to increase the cell growth index. Accordingly, it can be concluded that aeration in this system did not lead to shear damage, but caused the cell growth to decrease by affecting the concentration of elements in the culture medium through increasing evaporation. There are several reports on the effect of aeration on cell biomass and production of metabolites in bioreactors. In an overview
on reported results, it can be stated that although decreased aeration can be an inhibitor for cell growth, the increased aeration also caused shear damage and browned the cells. In most of them, aeration has been reported as a suitable factor for plant cell growth (Jeong and Park 2005; Dong et al. 2013) that confirm the obtained results of this study. (Rao and Ravishankar 2002). Dong et al (2013) showed that the aeration in the range of 0.1 to 0.4 VVM in bioreactor culture of ginseng suspension cells increases the cell biomass, while over 0.5 VVM will reduce it. Therefore, each plant has a certain threshold of aeration which needs to be optimized.

On the other hand, although the effect of aeration on cell biomass was improved by keeping the volume of the medium on a certain value, the results of this study revealed that aeration could not stimulate the production of crocin in the proliferated cells or caused crocin to degenerate due to supplying oxygen. So far, no clear relationship has been pointed out between the 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 cultures. 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, aeration in bioreactor which is strongly influenced by plant species and the secondary metabolites intended to be produced, and needs to be optimized due to the specific conditions. Given the fact that, crocin is very unstable when exposed to oxygen (Rahaiee et al. 2015), in this study, aeration in stirred bioreactor has probably led to the destruction of crocin.

**Conclusion**

Although, in this study, MES in applied concentrations did not show the buffering properties in the saffron cell suspension cultures, it was found that it was able to stimulate the cell biomass and crocin production to a higher level as compared to the control. Gradual supplying of sucrose to the concentration of 6% has also a positive effect on the cell growth index and crocin production. It was also revealed that the use of acid and base to prevent the pH fluctuation in tested bioreactor system, not only inhibited the cell growth, but also led to cells browning and death. Keeping the pH constant caused also degradation of crocin or inhibition of crocin synthesis. It is therefore concluded that in saffron cell suspension culture, the normal pH fluctuation in the medium is suitable for increasing cell biomass and crocin production. If aeration is associated with constant volume of the culture medium, it will have a positive effect on the cell growth index. However, in any case, aeration at the tested value (0.5 vvm), led to crocin not being detectable. Therefore, to find out the real effect of aeration on cell biomass and its relation to crocin production, the investigation on the lower levels of aeration is highly recommended.

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**Data availability** The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

**Declarations**

**Conflict of interest** Authors hereby declare that there is no conflict of interest.

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