

USE OF MULBERRY POMACE AS SUBSTRATE FOR CITRIC ACID PRODUCTION BY *Aspergillus niger* MT-4

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Abstract: Mulberry pomace (MP) is a waste material obtained after the production of pekmez, a traditional Turkish food. This study was performed to test the usability of MP as substrate for citric acid (CA) production by *Aspergillus niger* MT-4 for the first time. In the study, some culture conditions were also optimized to increase CA production in MP-based medium. Moisture, total carbohydrate, water-soluble carbohydrate, protein, lipid and ash contents of MP were determined as 31.1, 47.1, 1.3, 13.4, 1.8 and 1.4%, respectively. Experiments were carried out in 250 mL flasks containing 100 mL of production medium. Optimal MP concentration for both fungal biomass (FB) and CA production was determined as 120 g/L. All concentrations of KH_2PO_4 added to MP-based medium were found to decrease CA production but increase FB production. Optimal concentrations of MgSO_4 and $(\text{NH}_4)_2\text{SO}_4$ for CA production were found as 1 and 2 g/L, respectively. The other optimal parameters were determined as an initial pH of 7.0 and an incubation period of 5 days. Under the optimized culture conditions, the amount of CA produced was determined as 24.6 g/L. On day 5, Yp/s, Yp/x and Yx/s were calculated as 0.2 g CA/g MP, 1.43 g CA/g FB and 0.14 g FB/g MP, respectively.

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Özet: Dut posası (DP), geleneksel bir Türk yiyeceği olan pekmez'in üretiminden sonra ortaya çıkan bir atık maddedir. Bu çalışma, *Aspergillus niger* MT-4 ile sitrik asit (SA) üretimi için DP'nin substrat olarak kullanılabilirliğini test etmek için gerçekleştirilmiştir. Çalışmada ayrıca, SA üretimini artırmak için bazı kültür koşulları da optimize edilmiştir. MP'nin nem, toplam karbonhidrat, suda-çözünür karbonhidrat, protein, lipid ve kül içerikleri sırasıyla %31,1; 47,1; 1,3; 13,4; 1,8 ve 1,4 olarak belirlenmiştir. Deneyler, üretim besiyerinin 100 ml'sini içeren 250 ml'lik erlenler içerisinde gerçekleştirilmiştir. Hem fungal biyokütle (FB) hem de SA üretimi için optimum DP konsantrasyonu, 120 gr/lt olarak belirlenmiştir. DP bazlı ortama eklenen tüm KH_2PO_4 konsantrasyonlarının SA üretimini azalttığı fakat FB üretimini artırdığı belirlenmiştir. SA üretimi için MgSO_4 ve $(\text{NH}_4)_2\text{SO}_4$ 'ün optimal konsantrasyonları sırasıyla 1 ve 2 gr/lt olarak bulunmuştur. Diğer optimal parametreler, başlangıç pH'sı 7,0 ve inkübasyon süresi 5 gün olarak belirlenmiştir. Optimize edilmiş kültür koşulları altında, üretilen SA miktarı 24,6 gr/lt olarak belirlenmiştir. Beşinci günde, Yp/s; Yp/x ve Yx/s sırasıyla 0,2 gr SA/gr DP; 1,43 gr SA/gr FB ve 0,14 gr FB/gr DP olarak hesaplanmıştır. DP'nin SA dahil mikrobiyal metabolitlerin üretimi için fermentasyon substratı olarak kullanılabilirliği ilk kez bu çalışmada test edilmiştir.

Introduction

Mulberry belongs to the *Morus* L. genus of the Moraceae family. The genus includes 24 species and one subspecies. *M. alba* L., *M. nigra* L. and *M. rubra* L. are the main species grown in Turkey. Mulberry cultivation in Turkey has long been known dating back to 400 years ago (Ercisli & Orhan 2007).

Mulberry fruits in Turkey are consumed as fresh or fresh fruits are processed to prepare traditional products such as pekmez, pestil and köme. Pekmez, which is consumed mainly in breakfast is made from different

fruits but grape and mulberry are the most common fruits used in the process (Gunes & Cekic 2004, Cakmakci & Tosun 2010). For pekmez production, mulberry fruits are boiled in water until the sugars and other organic substances are passed into the water. After the boiling process, the mixture is filtered and the liquid fraction obtained is used for pekmez production. The remaining non-degrading solid fraction is referred to as pomace. Mulberry pomace (MP) is used as animal feed additives and no other use in Turkey is known. MP has also no important use in any application in the world.



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Citric acid (CA) is an organic acid commonly used in the food, pharmaceutical, cosmetic, detergent, chemical and textile industries (Dhillon *et al.* 2011, Torrado *et al.* 2011). Most of CA is used in food (about 70%) and pharmaceutical (12%) industries (Darouneh *et al.* 2009). The production of CA is carried out by extraction from plants or by chemical synthesis and microbial fermentation but about 99% of the production is achieved via microbial fermentation (Taskin *et al.* 2013, Arslan *et al.* 2016). Several microorganisms such as bacteria, filamentous fungi and yeasts are capable of producing CA. For example, filamentous fungi such as *Aspergillus niger* van Tieghem, *A. wentii* Wehmer, *A. foetidus* Thom & Raper and *A. carbonarius* (Bainier) Thom and *Trichoderma viride* Pers. and *Mucor pyriformis* Scop. have been reported to be good producers of CA. Similarly, yeast species belonging to different genera such as *Candida* Berkhout, *Yarrowia* Van der Walt & Arx, *Pichia* E.C. Hansen, *Hansenula* Syd. & P. Syd. have been documented to be potential producers of CA (Soccol *et al.* 2006, Show *et al.* 2015). Despite the variety of these different producers, microbial production of CA is mainly performed using *A. niger* since this filamentous fungus is able to use numerous cheap raw materials and to accumulate CA with higher yields (Soccol *et al.* 2006, Angumeenal & Venkappayya 2013, Taskin *et al.* 2013, Arslan *et al.* 2016). Microbial CA production is achieved by three different fermentation techniques as surface fermentation, submerged fermentation and solid-state fermentation. Submerged fermentation is performed using shaking flasks or fermenters. Shaking flask technique is usually used for the optimization of fermentation conditions (Show *et al.* 2015). CA production is affected by operational culture conditions such as pH, temperature, oxygen, incubation time, substrate concentration, minerals, carbon and nitrogen sources. In particular, high carbon source concentrations under the nitrogen and phosphorus-limited conditions are known to significantly increase CA synthesis (Soccol *et al.* 2006, Darouneh *et al.* 2009, Show *et al.* 2015, Arslan *et al.* 2016). Therefore, optimization of culture conditions in CA production is considered an important criterion.

Growth substrates make up the major part of the production costs in fermentation studies. Therefore, selection of a low-cost substrate is considered as a major aspect in microbial fermentation studies (Taskin *et al.* 2013). For example, with the aim of reducing the production cost in the production of CA, cheap agricultural wastes and/or byproducts such as beet molasses, black strap molasses, cane molasses, *n*-paraffin, glycerol, whey and waste oil are usually preferred as substrate (Soccol *et al.* 2006, Torrado *et al.* 2011; Show *et al.* 2015, Arslan *et al.* 2016). However, to our best knowledge, there is no study on the use of MP as a substrate in microbial fermentations. Therefore, the present study was performed to produce CA from *A. niger* MT-4 in shaking flask culture using MP as substrate and to optimize some culture conditions for enhancement of CA production.

Materials and Methods

Microorganism, materials and chemicals

Aspergillus niger MT-4 was obtained from Professor Mesut Taskin from the Department of Molecular Biology and Genetics, Atatürk University, Turkey (Taskin *et al.* (2013). MP was obtained from a commercial company producing mulberry pekmez in Erzurum province (Turkey). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Merck (Germany). The other chemicals (Tween 80, phenol, sulfuric acid, acetylacetone, Ehrlich reagent, N-acetylglucosamine, citric acid, pyridine and acetic anhydride) were purchased from Sigma (USA).

Preparation of spore suspension

The fungal culture was left to sporulation at 30°C for 10 days on the slant containing PDA medium. At the end of this period, conidia were suspended in sterile physiological water including a surfactant (0.2 mL/L Tween 80). The slant was vortexed for approximately 5 min to distribute the spores homogeneously. The prepared suspension was then filtrated through three layers of muslin to eliminate hyphae and unsuspended conidia. The spore concentrations were determined by using a haemocytometer and the final spore count was adjusted to 10⁶ spores per mL. During the experiments, 1 mL of the prepared spore suspension was used for inoculation of the production medium containing MP.

Determination of chemical composition of MP

The moisture content of MP was determined by the weight difference before and after drying in a oven for about 24 h at 100°C, up to constant weight. Total nitrogen content was determined using a micro-Kjeldahl apparatus, and the protein content was estimated by multiplying the nitrogen content by 6.25. Ash content was determined by combusting MP for 3 h in a muffle furnace (Thermolyne 62 700, Barnstead/Thermolyne Corp., Dubuque, IA, USA) at 550°C. The total lipid content was determined according to the Soxhlet extraction method using diethyl ether as solvent. Water-soluble carbohydrate and total carbohydrate contents were determined using phenol-sulfuric acid method (Dubois *et al.* 1956). For analysis of water-soluble carbohydrates, MP was boiled in water for about 5 min. The suspension was then centrifuged to remove solid particles. Finally, the analysis of water-soluble carbohydrates in the obtained liquid fraction (supernatant) was performed by the phenol-sulfuric acid method.

CA production in mulberry pomace-based medium

CA production using *A. niger* MT-4 was performed in 250 mL flasks containing 100 mL of sterile production medium. The production medium (pH 6.0) was prepared by adding MP into distilled water. Optimization of fermentation parameters was performed by using one-factor-at-a-time method. Initial experiments were performed to determine the most favorable concentration of dried MP. For this purpose, different concentrations of

dried MP from 30 to 150 g/L were tested. The effects of different concentrations of KH_2PO_4 (0-1.5 g/L), MgSO_4 (0-2.0 g/L) and $(\text{NH}_4)_2\text{SO}_4$ (0-4 g/L) on CA production in MP-based medium were tested. Then, different initial pHs (pH 2-8) and incubation times (2-7 days) were tested to increase CA production in MP-based medium. All the experiments were performed at 30°C on a shaking incubator at 150 rpm.

Analytical methods

Final pH of the culture and the initial pH of the MP-based medium were measured using a pH meter (Ohaus Starter 3100). 10 mL sample taken from the culture after an appropriate cultivation was centrifuged at 5000 rpm for 5 min. The obtained supernatant was used for CA analysis. The concentration of CA was determined according to the acetic anhydride method (Marier & Boulet 1958). In brief, 1 mL of supernatant was first mixed with 1.3 mL of pyridine. Then, 5.7 mL acetic anhydride was added into the samples. After incubation at 32°C for 30 min, absorbance of CA was determined spectrophotometrically at 410 nm. Anhydrous CA (50-300 µg/mL) was used as standard. In case of very intense color formation, samples were diluted with distilled water. CA content of the samples was determined according to the standard graph prepared with anhydrous CA.

During the incubation period, some of the MP was not used as a substrate by the fungus. This part, which was not used as a substrate and remained in the culture, was named non-fermented MP. At the end of the cultivation period, it was observed that the non-fermented MP was attached to the fungal biomass (FB) and not separated from it by centrifugation. Therefore, the solid fraction (non-fermented MP + FB) consisting of non-fermented MP and FB was named as total biomass (TP).

For the determination of FB amount in TP, N-acetylglucosamine content in TP was measured. In brief, 1 mL of concentrated H_2SO_4 was added to 0.5 g of TP. Following this, acetylacetone reagent (1 mL) was added to the mixture. The mixture was incubated in a boiling water bath for 20 min and then cooled at room temperature. Then, 6 mL of ethanol and 1 mL of Ehrlich reagent were added to the cooled mixture. The final mixture was again left to incubation at 65 °C for 10 min and then cooled at the room temperature. Finally, optical density (OD) of the final mixture was determined at 530 nm (Velmurugan *et al.* 2011). The obtained absorbance was then used for the calculation of FB produced in MP-based medium. For this purpose, the following simple equation was used.

$$\text{FB} = (\text{A1}/\text{A2}) \times \text{TB}$$

A1: The absorbance assayed for N-acetylglucosamine content in per g of the dried TB (TB; FB + non-fermented MP) in MP-based medium at the end of cultivation period.

A2: The absorbance assayed for N-acetylglucosamine content in per g of dried FB, which was produced in standard PDB medium at the end of cultivation period.

TB: Total biomass (FB + non-fermented MP) in MP-based medium at the end of cultivation period.

Statistical analysis

Each analysis was repeated at least three times in two replicates. Statistical difference was analyzed with one-way ANOVA in the SPSS 15.0 package program at $P < 0.05$ significance level.

Results

Chemical composition of MP

The moisture content of raw MP was determined as 31.1%. Total carbohydrate, protein, lipid and ash contents of MP were determined as 47.1, 13.4, 1.8 and 1.4%, respectively. The ratio of water-soluble carbohydrates was found to be 1.3%.

Table 1. Chemical composition of MP.

Components	Content (%)
Moisture	31.1±1.90
Protein	13.4±1.04
Lipids	1.8±0.17
Ash	1.4±0.12
Total carbohydrate	47.1±2.72
Water-soluble carbohydrate	1.3±0.10

All measurements are mean ± standard deviations (±SD) of six determinations (n = 6).

Optimization of CA production from *A. niger* in MP-based medium

In the first step of the study, different concentrations of dried MP were tested for production of CA and FB from *A. niger* MT-4. Increased MP concentration increased both CA synthesis and fungal growth, and maximum concentrations of CA (10.6 g/L) and FB (14.3 g/L) were reached in medium containing 120 g/L MP (Fig. 1). Based on these results, the following experiments were performed on medium containing 120 g/L MP.

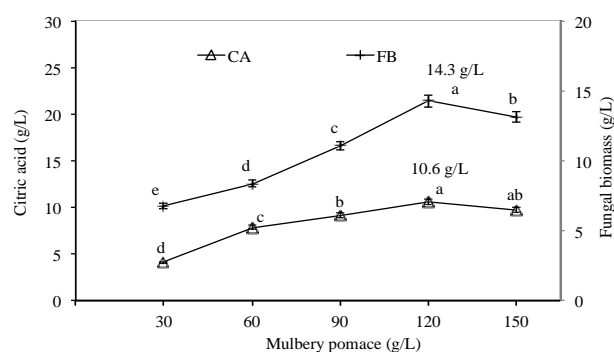


Fig. 1. Effect of MP on CA synthesis and fungal growth in *A. niger* MT-4. Culture conditions: initial pH 6.0, temperature 30°C, shaking speed 150 rpm and incubation time 4 days. All the measurements were mean ± standard deviations (±SD) of six determinations (n = 6). Different letters in the line of CA and FB indicate significant differences ($P < 0.05$).

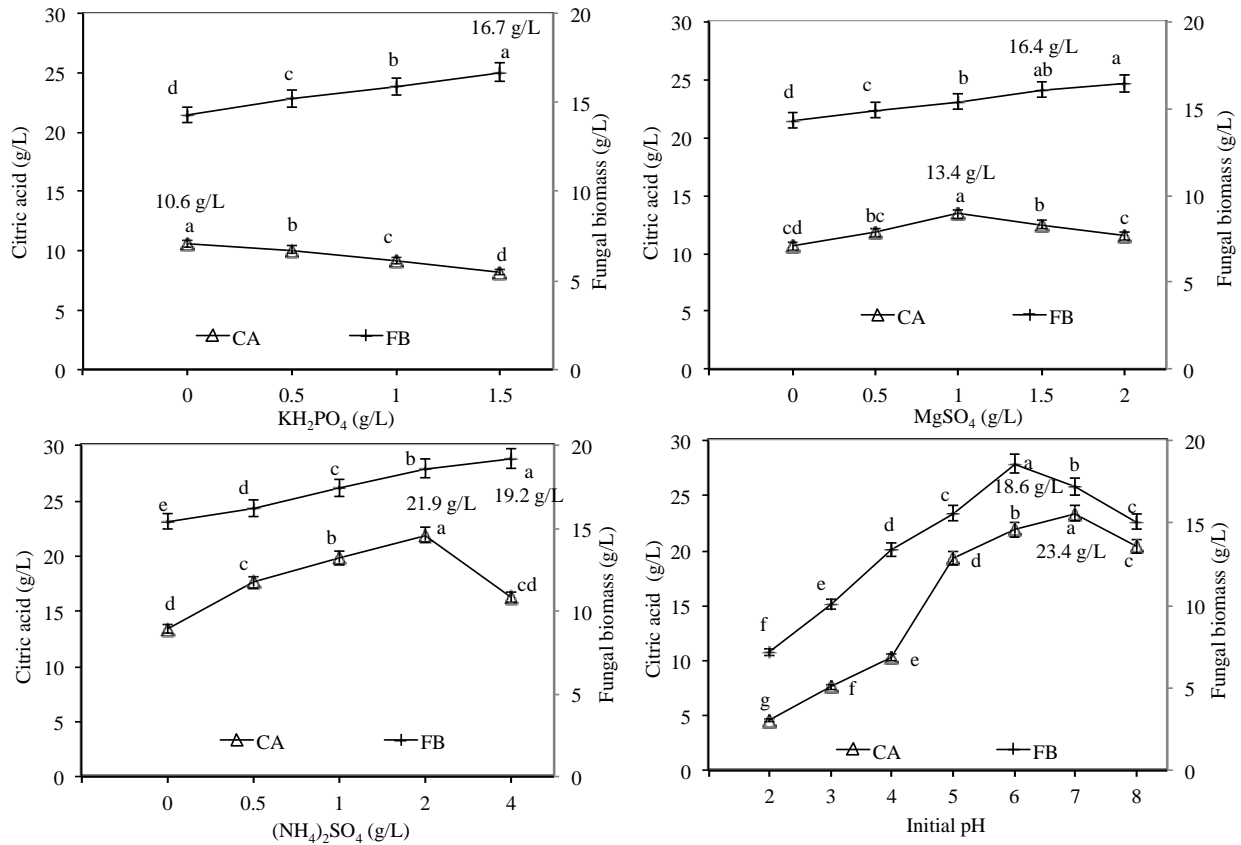


Fig. 2. Effect of KH_2PO_4 , MgSO_4 , $(\text{NH}_4)_2\text{SO}_4$ and initial pH on citric acid synthesis and fungal growth in *A. niger* MT-4. Culture conditions for optimization of KH_2PO_4 concentration: MP concentration 120 g/L and initial pH 6.0. Culture conditions for optimization MgSO_4 concentration: MP concentration 120 g/L, KH_2PO_4 0 g/L and initial pH 6.0. Culture conditions for optimization of $(\text{NH}_4)_2\text{SO}_4$ concentration: MP concentration 120 g/L, KH_2PO_4 0 g/L, MgSO_4 1 g/L and initial pH 6.0. Culture conditions for optimization of initial pH: MP concentration 120 g/L, KH_2PO_4 0 g/L, MgSO_4 1 g/L and $(\text{NH}_4)_2\text{SO}_4$ 2 g/L. All experiments were performed at 30°C and 150 rpm for 4 days. All the measurements are mean \pm standard deviations (\pm SD) of six determinations ($n = 6$). Different letters in the line of CA and FB indicate significant differences ($P < 0.05$).

After determination of the optimum concentration of MP, the effect of different concentrations of KH_2PO_4 on cell growth and CA synthesis was investigated. The maximum CA production was achieved in the control medium (KH_2PO_4 -free medium), whereas FB reached to the maximum (16.7 g/L) when 1.5 g/L KH_2PO_4 was added to MP-based medium (Fig. 2). Considering these results, the following experiments were carried out in the medium, which was not supplemented with KH_2PO_4 . All tested concentrations of MgSO_4 increased fungal growth (Fig. 2). In contrast to fungal growth, the maximum CA production (13.4 g/L) was achieved in the medium supplemented with 1 g/L MgSO_4 . However, excessive concentrations of MgSO_4 gradually decreased CA production. For example, CA production decreased up to 11.5 g/L when 2 g/L MgSO_4 was added to the medium. Therefore, the subsequent experiments were performed in the medium supplemented with 1 g/L MgSO_4 . The experiments revealed that 2 g/L $(\text{NH}_4)_2\text{SO}_4$ caused maximum CA production (21.9 g/L) but higher $(\text{NH}_4)_2\text{SO}_4$ concentrations decreased CA production. In contrast to CA synthesis, all tested concentrations of $(\text{NH}_4)_2\text{SO}_4$ increased fungal growth and the maximum FB (19.2 g/L) was reached at the highest $(\text{NH}_4)_2\text{SO}_4$ of 4 g/L

(Fig. 2). Taking into account these results, an ammonium sulfate concentration of 2 g/L resulting in the maximum CA production was chosen for subsequent experiments.

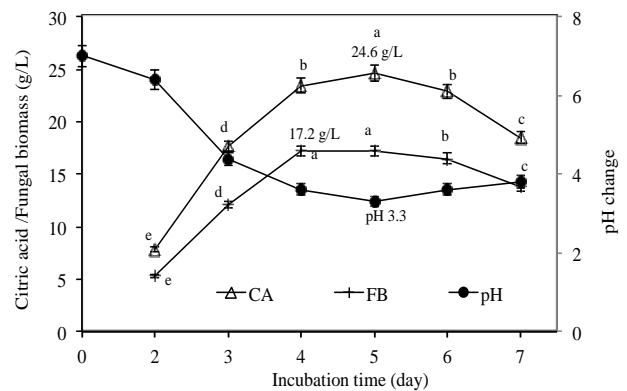


Fig. 3. Effect of incubation time on cell growth, CA synthesis and pH change in MP-based medium. Culture conditions: MP concentration 120 g/L, KH_2PO_4 0 g/L, MgSO_4 1 g/L, $(\text{NH}_4)_2\text{SO}_4$ 2 g/L, initial pH 7.0, temperature 30°C, shaking speed 150 rpm. All the measurements are mean \pm standard deviations (\pm SD) of six determinations ($n = 6$). Different letters in the line of CA and FB indicate significant differences ($P < 0.05$).

When the effect of initial pH on CA synthesis and fungal growth was investigated, it was determined that maximum CA synthesis (23.4 g/L) occurred at an initial pH of 7.0, but FB reached to the maximum (18.6 g/L) at pH 6.0 (Fig. 2). Based on this result, the following experiments were carried out in the medium with an initial pH of 7.0.

The highest increase in CA production occurred in the first 4 days of incubation and CA concentration reached to the maximum (24.6 g/L) on day 5 (Fig. 3). FB reached the highest value (17.2 g/L) on day 4 and remained stable on day 5. But, reductions in both FB and CA concentrations were observed after the 5th day. For example, at the end of day 7, FB and CA concentrations were measured as 13.8 and 18.5 g/L, respectively. Since maximum productions of CA (24.6 g/L) and FB (17.2 g/L) were achieved at 120 g/L MP concentration, Yp/s (gram CA produced/gram substrate), Yp/x (gram CA produced/ gram FB produced) and Yx/s (gram FB produced/gram substrate) were calculated as 0.2 g CA/g MP, 1.43 g CA/g FB and 0.14 g FB/g MP, respectively. When initial pH was adjusted at 7.0, there was a continuous decrease in culture pH up to the end of 5th day, but an increase in culture pH was observed again after 5th day. Lowest pH value (pH 3.3) was detected on day 5, by which the maximum CA concentration was reached.

Discussion

The previous studies demonstrated that hydrolysates, which are prepared from agricultural wastes and/or byproducts using chemical or enzymatic hydrolysis processes can be utilized as substrates for CA production in the culture of *A. niger* strains (Watanabe *et al.* 1998, Hu *et al.* 2014, Faruk *et al.* 2014, Muna *et al.* 2018). But, it is well known that when chemicals and/or enzymes are used for the preparation of hydrolysates, the production cost of CA increases. Therefore, researchers suggested using *Aspergillus* strains in CA production, which are capable of secreting hydrolytic enzymes (amylase, cellulase, pectinase etc.) and thereby growing directly on agricultural wastes and/or by-products (Andersen *et al.* 2011, Afify *et al.* 2012, Rehman *et al.* 2014). However, the potential of *A. niger* to produce CA on MP, which is not subjected to a pretreatment process such as chemical or enzymatic hydrolysis, has not been studied yet.

The chemical analyses revealed that dried MP contained very low amount of water-soluble carbohydrates. This situation can be attributed to the boiling of mulberry fruits at high temperature for a long time during the production of pekmez (molasses). In other words, since water-soluble carbohydrates such as sucrose and glucose in mulberry fruits passed into pekmez, there was rather low soluble carbohydrate in MP. On the contrary, the experiments showed that the total carbohydrate content of MP was high. These carbohydrates may include pectin, starch, cellulose and soluble sugars. Considering that *A. niger* strains have hydrolytic enzymes, it is possible that insoluble carbohydrates such as pectin, starch and cellulose in MP

can be used as carbon source by *A. niger* MT-4. Besides, it was determined in this study that MP was rich in ash. Considering the knowledge that ash consists of macro and micro-elements, it is possible to say that MP can also be used as a mineral source by this filamentous fungus. The experiments also indicated that MP contained protein and therefore it may be used as a nitrogen source in the medium.

Usability of non-pretreated MP as a substrate for CA production from *A. niger* MT-4 was tested in this study. In the first stage, MP was used as a sole source of all nutritional factors (minerals, carbon and nitrogen source) for production of FB and CA. The maximum CA and FB production were achieved in medium containing 120 g/L MP, while higher MP concentrations decreased both CA and FB production. This result might be attributed to the increase in solid/liquid ratio of the production medium. Namely, high solid ratio (high MP concentration) might have prevented the homogeneous mixing of the substrate and sufficient oxygen intake into the culture. Since oxygen is particularly important for CA synthesis (Max *et al.* 2010), the decrease in oxygen concentration might have limited CA synthesis in MP-based medium.

The experiments revealed that when KH_2PO_4 was used as phosphorus (P) and potassium (K) source, all concentrations caused inhibition on CA synthesis but a continuous increase in fungal growth. These results should not be so surprising, because it has been reported that excessive P concentrations can limit CA synthesis but increase fungal growth in *A. niger* (Jernejc *et al.* 1982, Chen 1996, Vandenberghe *et al.* 1999, Max *et al.* 2010, Angumeenal & Venkappayya 2013). These results indicate that P content of MP was sufficient to promote CA synthesis and no additional P source is required in MP-based medium for CA synthesis. When MgSO_4 was added into the medium, its concentrations ≤ 1 g/L promoted CA synthesis but higher concentrations caused a slight inhibition. This finding is good agreement with the fact that although MgSO_4 has a beneficial effect on CA synthesis in *A. niger*, its excessive concentrations can inhibit CA synthesis (Vandenberghe *et al.* 1999, Ikram-ul *et al.* 2004).

It has been documented that CA production is directly influenced by the nitrogen source, and $(\text{NH}_4)_2\text{SO}_4$ is a good nitrogen source for CA production (Vandenberghe *et al.* 1999, Max *et al.* 2010). Considering this knowledge, the present experiments also focused on investigating the effect of $(\text{NH}_4)_2\text{SO}_4$ as additional nitrogen source on CA production in MP-based medium. The experiments showed that CA production could be achieved in MP-based medium without $(\text{NH}_4)_2\text{SO}_4$ (additional nitrogen source). This can be explained by the use of proteinous compounds in MP as nitrogen source in the medium. However, it was seen that $(\text{NH}_4)_2\text{SO}_4$ concentrations ≤ 2 g/L caused increases in CA synthesis. This result revealed that the nitrogen in MP was insufficient for the production of high amounts of CA and additional nitrogen was required in the medium. The results also showed that

(NH₄)₂SO₄ concentrations over 2 g/L decreased CA synthesis in the culture. Conversely, there was a continuous increase in FB even at the highest (NH₄)₂SO₄ of 4 g/L. This finding is in good agreement with the fact that excessive nitrogen concentration increases fungal growth but decreases the amount of CA produced (Hang *et al.* 1977, Vandenberghe *et al.* 1999, Soccol *et al.* 2006, Auta *et al.* 2014, Arslan *et al.* 2016).

The results showed that initial pH values between 5 and 7 were more suitable for CA and FB production. Especially, initial pH of 7.0 was the superior for CA production. It was reported that although acidic pHs such as pH 2 and 3 are more suitable for CA synthesis in *A. niger*, the germination of *A. niger* spores requires pH > 5 (Soccol *et al.* 2006). Namely, the initial pH of the culture for CA synthesis in *A. niger* should be adjusted to pH levels > 5. Therefore, it should not be surprising that initial pHs of 5-7 lead to more CA synthesis in *A. niger* MT-4.

The final experiments showed that FB and CA reached to maximum concentrations on days 4 and 5, respectively. The maximum increases in concentrations of both CA and FB were achieved in the first four days of fermentation. These results are similar to those reported in previous studies (Kareem *et al.* 2010, Taskin *et al.* 2013, Dienye *et al.* 2018) showing that incubation time affects cell growth and CA synthesis in *A. niger*. On day 5, CA concentration and CA yield (Yp/s) were determined as 24.6 g/L and 0.2 g CA/g MP, respectively. The CA yield achieved in submerged culture was similar to those reported in

previous studies (Alben & Erkmen 2004, Guc & Erkmen 2017).

The reduction in fungal biomass after day 5 may be attributed to cell lysis due to the depletion of nutritional compounds. The most likely cause of the decrease in CA concentration after day 5 may be the degradation of CA by the enzymes released into the culture when the fungal cells are lysed. A continuous drop in culture pH up to the end of the 5th day could be ascribed to the production of organic acids, especially CA. Similar decreases in culture pH during CA production were also reported in the previous studies (Ali *et al.* 2002, Dienye *et al.* 2018). Increment in culture pH after the 5th day might be attributed to the releasing into the culture of some alkaline compounds due to fungal cell lysis.

In conclusion, this study revealed that MP alone could be used as a complex substrate for CA production with *A. niger* MT-4, but more CA production could be achieved when MP-based medium was supplemented with (NH₄)₂SO₄ and MgSO₄ at suitable concentrations. On the other hand, optimization of initial pH and incubation time could increase CA production in MP-based medium. When the optimal culture parameters are selected, 24.6 g/L CA could be produced in MP-based medium. The use of MP in CA production will contribute to the reduction of both fermentation cost and environmental pollution. In future studies, MP can be also tested as a substrate in the culture of microorganisms in the production of other substances such as lactic acid, acetic acid, single cell protein, ethanol, pigment and polysaccharide.

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