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ORIGINAL PAPER

Citric Acid Production by *Aspergillus niger* from Agro-Industrial By-Products: Molasses and Chicken Feather Peptone

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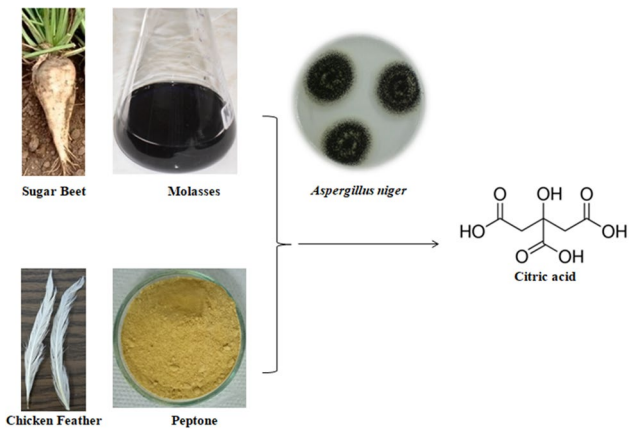
Abstract

Citric acid is a commercially important organic acid with a wide range of applications. To reduce the cost of producing citric acid, sugar beet molasses and chicken feather peptone (CFP) were used as the sole carbon and nitrogen sources, respectively for submerged citric acid biosynthesis using *Aspergillus niger*. To improve the citric acid production, the parental isolate of *A. niger* MO-25 was improved by mutation using ethidium bromide. Citric acid production using molasses was significantly affected by CFP concentrations (1–6 g/L). The maximum citric acid concentration was determined at 4 g/L CFP and 168 h. When CFP compared to commercial peptones (casein and bacto), the highest citric acid production was obtained with CFP. Furthermore, the addition of KH_2PO_4 (0.15 g/L) enhanced citric acid production (68.8 g/L). These results suggested that sugar beet molasses supplemented with CFP as organic nitrogen and mineral salt sources could be utilized for the economical and efficient production of citric acid. This is the first study to investigate the influence of CFP for citric acid production.

Graphical Abstract



Keywords *Aspergillus niger* · Chicken feather peptone · Citric acid · Molasses

Introduction

Citric acid ($C_6H_8O_7$) is an intermediate product of the Krebs cycle. Because of its numerous applications in various industries (food, pharma, agriculture, textile), the volume of citric acid production by fermentation is continually on the increase [1]. Every year, over two million tons of citric acid is produced with fermentation because the chemical synthesis of citric acid is more expensive

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than fungal fermentation [2]. *Aspergillus niger* and *Yarrowia lipolytica* are well known producers of citric acid from agro-industrial wastes. However, *Y. lipolytica* produces significant amount of unwanted by-product, isocitric acid. *A. niger* is the most commonly used fungus to produce citric acid because of its relatively high yield, ease of harvesting and ability of using a variety of raw materials [3, 4].

Citric acid production by *A. niger* is strongly influenced by the composition of the medium (the type and concentration of the carbon, nitrogen, phosphate and trace elements), the pH of the fermentation medium, oxygen, the morphology of the citric acid producing microorganism, and the fermentation method [3, 5–8].

The improvement of microbial strains for the over-production of microbial products has attracted attention in the fermentation process. For this purpose, mutagenic agents (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine, ethyl-methane sulphonate, ethidium bromide and ultraviolet radiation) have been applied to *A. niger* strains to improve citric acid production [9–11]. Chemical mutagenic agents such as ethidium bromide [12], *N*-methyl-*N*-nitro-*N*-nitrosoguanidine [13] and NTG [14] were found to be better mutagenic than UV for citric acid production.

Different hydrolytic enzymes are generally produced by *A. niger* [11, 15]. For this reason, various agro-industrial by-products or wastes (molasses, apple pomace, pineapple waste, cassava baggasse, banana peel and kiwi fruit peel) have demonstrated high potentials for the commercial production of citric acid [16–18]. The production of citric acid using cheap and renewable carbon and nitrogen sources from agro-industrial by products provides considerable advantage combined in the benefit of waste management as well as decrease in the cost of citric acid production [16]. Molasses is a by-product obtained from the sugar industry, and it is commonly used as carbon source for the production of biotechnological products (e.g. enzymes, organic acids, pigments, polysaccharides). It also contains valuable compounds for the fermentation process like amino acids, minerals and organic compounds [19]. Poultry-processing plants generate million tons of chicken feathers annually worldwide as a bioorganic waste product. Since feathers are discarded as waste and degraded very slowly in nature, they may become an environment problem [20, 21]. Feathers, which constitute up to 10% of total chicken weight, are composed of over 90% protein composed of keratin [21–23]. Keratin is a source of valuable amino acids and keratin wastes can be hydrolyzed with acid or enzymatic hydrolysis for the use of microorganisms [21, 24]. This paper aims to contribute to the use of agro-industrial wastes (molasses and chicken feather) as substrates for the production of citric acid.

Materials and Methods

Preparation of Chicken Feather Peptone

The chemicals used in this study were analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA) and Difco (Detroit, MI, USA). Chicken feathers were supplied by a chicken processing plant at Devrek, Zonguldak, Turkey. Chicken feathers were washed with deionized water and dried in oven at 60 °C. Dried feathers were cut into smaller pieces and then these smaller pieces were powdered with a blender. This material was hydrolysed by modifying the method of Kurbanoglu and Kurbanoglu [25], and the production process of CFP is shown in Fig. 1.

Isolation and Identification of *A. niger*

The wild-type strain of *A. niger* MO-25 was isolated from soil sample by the serial dilution method. Culture growing on PDA and Malt Extract Agar was identified according to microscopic observations such as morphological characters of mycelium and conidia [26]. Furthermore, genomic DNA was extracted from the isolate and the sequencing of the Internal Transcribed Spacers (ITS) regions, specifically the ITS1 and ITS2 non-coding regions flanking the 5.8S rDNA was used to determine the specie [27]. The ITS rRNA gene was sequenced and searched in GenBank. This strain was maintained on PDA slants at 4 °C.

Preparation of Conidial Inoculum

To prepare conidial suspensions, *A. niger* isolates were grown on PDA slants at 30 °C for 7 days. A spore suspension was prepared by adding 10 mL sterile distilled water containing 0.1% Tween-80. The spore suspensions were adjusted to 5×10^6–1×10^7 spores/mL using hemocytometer.

Mutagenesis Using Ethidium Bromide

The wild-type strain of *A. niger* MO-25 was used for strain improvement by mutation. Ethidium bromide (EB) was selected as the chemical mutagen. The diluted spore suspension (1×10^7 spores/mL) were treated with EB at 0.5 mg/mL and finally incubated at 28 °C. At the end of 30, 60, 90, 120 and 150 min. of incubation, the treated suspensions were washed three times with sterile saline water [12]. After the treatment of spores, 100 fold serials dilution of spores were arranged to give more or less 30–40 colonies per plate. In dark conditions, the treated suspensions (0.1 mL) were poured to the separate PDA

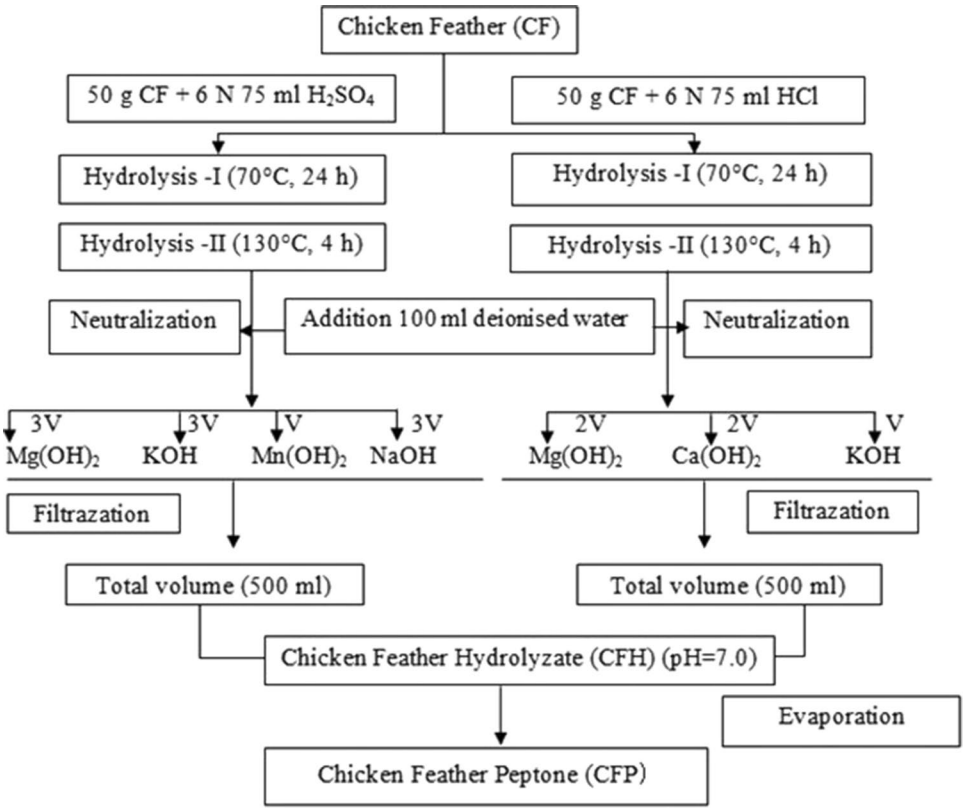

Fig. 1 A production scheme for CFP

plates containing 2% triton X-100 as colony restrictor. The untreated spore suspension was also plated as a control.

Screening of Cultures of *A. niger*

The *A. niger* mutants were screened qualitatively in petri plates containing Foster medium (5 g/L glucose, 1 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 15 g/L agar, pH 5) with bromocresol green (0.5 g/L) as an indicator [10]. *A. niger* mutant strains producing citric acid were selected on the basis of the Acid Unitage (AU) value. The amount of citric acid was related to the zone of color change. After incubation at 30 °C for 5 days, AU value was measured by the following formula: AU = DA/DC (DA: Diameter of acid zone, DC: Diameter of the colony). The mutant strains with high AU value were selected and transferred to PDA slants. Selected *A. niger* mutants were used for further screening by submerged fermentation.

Pretreatment of Molasses

Sugar beet molasses obtained from Erzurum Sugar Mill (Turkey). For potassium ferrocyanide ($K_4Fe(CN)_6$) treatment, the pH of the molasses solution containing 200 g/L of total sugars was adjusted to pH 5.5 with 1 N H_2SO_4 and the solution heated at 90 °C for 15 min. The hot liquid was treated with 0.1 g/L $K_4Fe(CN)_6$ to encourage the precipitation of heavy metals, allowed to stand for 24 h at room temperature and then centrifuged at 3000 rpm for 10 min.

Fermentation Medium

The screening experiments were performed in molasses-peptone medium containing treatment 150 g/L molasses sugar and 2 g/L CFP. To study effect of CFP on the citric acid production, chicken feather peptone medium (CFPM) contained the following: treatment 150 g/L molasses sugar and 1–6 g/L CFP. The pH of the media were adjusted to 6.0 with 1N H_3PO_4 and 1N NaOH before autoclaving at 121 °C for 15 min. The 200 ppm $K_4Fe(CN)_6$ solution was added before inoculation while the medium still hot. After cooling at room temperature, 1 mL of spore suspension was inoculated to 50 mL of medium in 250 mL flasks and incubated at 30 °C on a rotary shaking incubator at 200 rpm. Later, CFP was compared with two commercial peptones (bacto peptone [BP], casein peptone [CP]) at the optimal CFP concentration.

The influence of KH_2PO_4 and $MgSO_4$ on the citric acid production was also investigated. KH_2PO_4 was further optimized in the range of 0.05–0.25 g/L for improved citric acid production. The effect of $MgSO_4$ on the production citric acid was studied in the concentration range of 0–0.2 g/L.

Analytical Methods

Total sugar, dry matter and ash contents of the CFP were estimated by AOAC methods [28]. Total nitrogen and fat contents were determined by the Kjeldal method and Soxhlet extraction method, respectively. Amino acids in the CFP were analyzed as their pre-column derivatives by HPLC [29]. Citric acid was measured using pyridine–acetic anhydride method of Marrier and Boulet [30]. Briefly, pyridine and acetic anhydride were added to the diluted fermented broth with 1:1.3:5.7 ratios and after stirring, immediately placed in a water bath at 32 °C for 30 min. Citric acid concentrations were determined by spectrophotometer at 420 nm and by comparison with a standard curve. Reducing sugar was analyzed by the DNS (3,5-dinitrosalicyclic acid) method [31]. Biomass concentration was estimated by means of dry weight measurement. The yield of citric acid was calculated as following formula:

$$\text{Citric acid yield } (\%) = 100 \times \left[\text{citric acid (g/L)}/\text{consumed sugar(g/L)}\right]$$

Statistical Analysis

The media were compared against each other. The experiments were replicated three times in a randomized block design. All data were analyzed using the general linear models procedure of SPSS. Differences among means were tested for significance ($p < 0.05$) by Duncan's multiple range tests.

Results and Discussion

Production and Chemical Analysis of CFP

As shown in Fig. 1, 100 g chicken feathers were hydrolysed with acids (HCl and H_2SO_4), and then neutralized with NaOH, KOH, $Mg(OH)_2$ and $Ca(OH)_2$. The main chemical composition of CFP was shown in Table 1. CFP was found to be rich in both macro- and micronutrients. It was detected that CFP had high protein (56 g/100 g), ash (41.5 g/100 g) and low fat (0.2 g/100 g) contents. CFP contained all of amino acids, except methionine and tryptophan, at varying concentrations and was especially rich in alanine (3.758 g/100 g), leucine (5.019 g/100 g), glutamate (6.107 g/100 g), glycine (5.453 g/100 g), serine (4.250 g/100 g) and proline (8.106 g/100 g). Similar to this study, it has been reported that although animal feeds from chicken feathers are rich in glutamic acid, alanine, serine and leucine [32, 33]. The produced CFP has high nitrogen content (9 g/100 g), therefore it can be used as nitrogen source. As seen in Table 1, CFP was rich in especially N, Ca, K, Mg, S and Na because of the hydrolysis process. The chemical composition of peptone depends on hydrolysis and neutralization processes. The similar results were reported in the previous studies [34, 35]. These characteristics indicate that CFP important substrate for the production of many microbial products.

Table 1 The main chemical composition of CFP

Components	g/100 g	Amino acids	g/100 g
Nitrogen	9.0	Aspartate	3.07
Total protein	56	Glutamic acid	6.107
Total sugar	–	Asparagine	0.077
Fat	0.2	Serine	4.250
Ash	41.5	Histidine	0.302
Cu	0.2	Sarcosine	0.132
Zn	0.2	Proline	8.106
Fe	0.2	Hidoksil-L-proline	1.244
$MgSO_4$	1.8	Glycine	5.453
K_2SO_4	6.2	Threonine	0.010
$Na_2\ SO_4$	6.45	Sitrulin	0.216
$MgCl_2$	3.6	Arginine	1.174
$CaCl_2$	9.1	Alanine	3.758
$MnCl_2$	1.0	Tyrosine	0.846
KCl	4.6	Cysteine	0.817
		Valine	3.547
		Methionine	< 0.001
		Tryptophan	< 0.001
		Phenylalanine	2.756
		Isoleucine	2.513
		Ornithine	1.170
		Leucine	5.019
		Lysine	0.978

Citric Acid Production of the Wild-Type Strain and the Mutants

Aspergillus niger is very common species and it can be isolated from a wide variety of sources [26, 36]. In this study, *A. niger* MO-25 was isolated from soil. It was observed that the hyphae of *A. niger* were septate. The conidial heads were globose with an average diameter of 550 µm. Phialids borne on the metullae were fairly uniform. ITS rRNA gene sequence was applied to identify the strain MO-25, and it was identified as *A. niger*. The sequence of *A. niger* MO-25 was deposited in GenBank with the accession number KF939141.

Strain improvement is one of the promising approaches for increased production of industrially important metabolites by microorganisms. Many researchers reported that random mutation (radiation or chemical mutagens) and selection strategies (indicator dyes and 2-deoxy, D-glucose as selective marker) can be used for obtaining citric acid over producing isolates of *A. niger* [10, 37]. Strain improvement

of *A. niger* MO-25 was carried out by chemical mutagenesis (ethidium bromide, EB) to increase citric acid production. EB is a nucleic acid intercalating agent and frameshift mutagen. For this purpose, many researchers have been used this mutagen [38, 39]. The culture of *A. niger* MO-25 was treated with EB for different time intervals. With gradual increase in treatment time the number of survivors was decreased. Exposure of parent strain with EB for 150 min. gave 5.88% survivals rate.

Firstly, citric acid producer mutants were selected on bromocresol green indicator plates based on Acid Unitage (AU) value (Diameter of acid zone/Diameter of the colony) of a single colony. The halo formation resulted from the acid production in Foster medium, which was verified through the observation of a yellow halo formation (initially green), because of fall of pH (Table 2). Among the fifty-five mutants, EB-12 presented a DH/DC relation (1.82) higher than that presented by the wild (1.42) and the other mutants (1.30–1.79). Secondly, the mutants were screened for their ability to produce citric acid in molasses-peptone medium for 168 h (Table 2). In this medium, mutant EB-12 produced a maximum of 46.3 g/L of citric acid while its parent strain (MO-25) produced only 31.08 g/L of citric acid. As shown in Table 2, the mutant strain EB-12 gave 1.48-folds higher production of citric acid compared with the parent strain. For this reason, EB-12 mutant strain was used throughout the study.

The Effects of CFP on Citric Acid Production

Figure 2a depicts the effect of different CFP concentrations (1–6 g/L) on citric acid fermentation by the mutant *A. niger* EB-12, using $K_4Fe(CN)_6$ treated molasses (15.0%, w/v sugar) as a carbon source. It was found that addition of CFP into the molasses peptone medium had a promoting effect on the excretion of citric acid from mycelial cells. The maximum citric acid production (64.40 g/L) was obtained with CFP at a concentration of 4 g/L for 168 h. The biomass and sugar consumption were 22.41 and 140 g/L, respectively. The highest fermentation yield (45.7%) was obtained with CFP at the concentration of 4 g/L and the lowest fermentation yield (36.03%) was obtained with CFP at the concentration of 6 g/L. As seen in Fig. 4, with regard to citric acid yield, significant differences were obtained between media ($P < 0.05$). Citric acid production promoting effect of CFP may be related to the chemical composition of CFP. As seen in Table 1, CFP was observed to be rich in both amino acids and various salts. These nutrients have a positive effect on growth and citric acid production. Some amino acids (arginine, glutamate, glycine, aspartate, lysine and valine) enhance the production of citric acid [40–42]. Firstly, this increase can be explained with amino acids cause physiological acidity in the medium which is favourable for citric acid fermentation. Secondly, the citric acid cycle takes place in the mitochondria of the cell. Amino acids can enter at different steps (varies greatly from one amino acid to the other) in the citric acid cycle and they provides precursors for citric acid production [43]. For example, the aspartic acid and glutamic acid are degraded to oxaloacetate and α-ketoglutarate, respectively. It was reported that the presence of glutamic acid, aspartic acid and lysine stimulated citric acid production to the extent of 80, 77 and 62%, respectively [42]. Also, Ali et al. [41] found that glycine and arginine were suitable for citric acid production by co-culture consortia of *Aspergillus ornatus* and *Alternaria alternata*. CFP contains these amino acids at various concentrations. The positive effect of CFP on the citric acid production can be explained by the change in mycelial morphology (pellet shape and size) and increasing use of sugar. The positive effect of Mg^{2+} and Ca^{2+} in CFP might also be related to the increase of mycelial branching level which improves the performance of the citric acid production process [37, 44, 45]. Researchers have also reported such beneficial effects of Mg^{2+}, Ca^{2+} and Cu^{2+} in counteracting the Fe^{2+} effect [46]. Moreover, Pera and Callieri [45] reported that addition of $CaCl_2$ into fermentation medium increased the uptake of sugar. At lower CFP concentrations, less citric acid formed might be due to the lower supply of available nitrogen for fungal growth. Further increase in the concentration of CFP (beyond 4 g/L) decreased production of citric acid. This inhibitory effect may be due to high salt concentration, some toxic materials in CFP and, change of the C/N rate of culture medium [35, 47]. Since molasses are rich both macro- and micronutrients, molasses medium rarely need

Table 2 Screening of selected mutants of *A. niger* on indicator medium and in molasses peptone medium

Strain no.	Plate screening Acid unitage (AU)	Submerged fermentation Citric acid (g/L)
MO-25	1.44	31.08
EB-4	1.66	37.50
EB-5	1.74	39.40
EB-8	1.56	34.60
EB-9	1.77	41.56
EB-12	1.82	46.30
EB-13	1.67	37.65
EB-14	1.79	42.46
EB-26	1.79	41.14
EB-29	1.71	38.40
EB-30	1.75	39.50
EB-38	1.65	36.80
EB-39	1.62	36.10

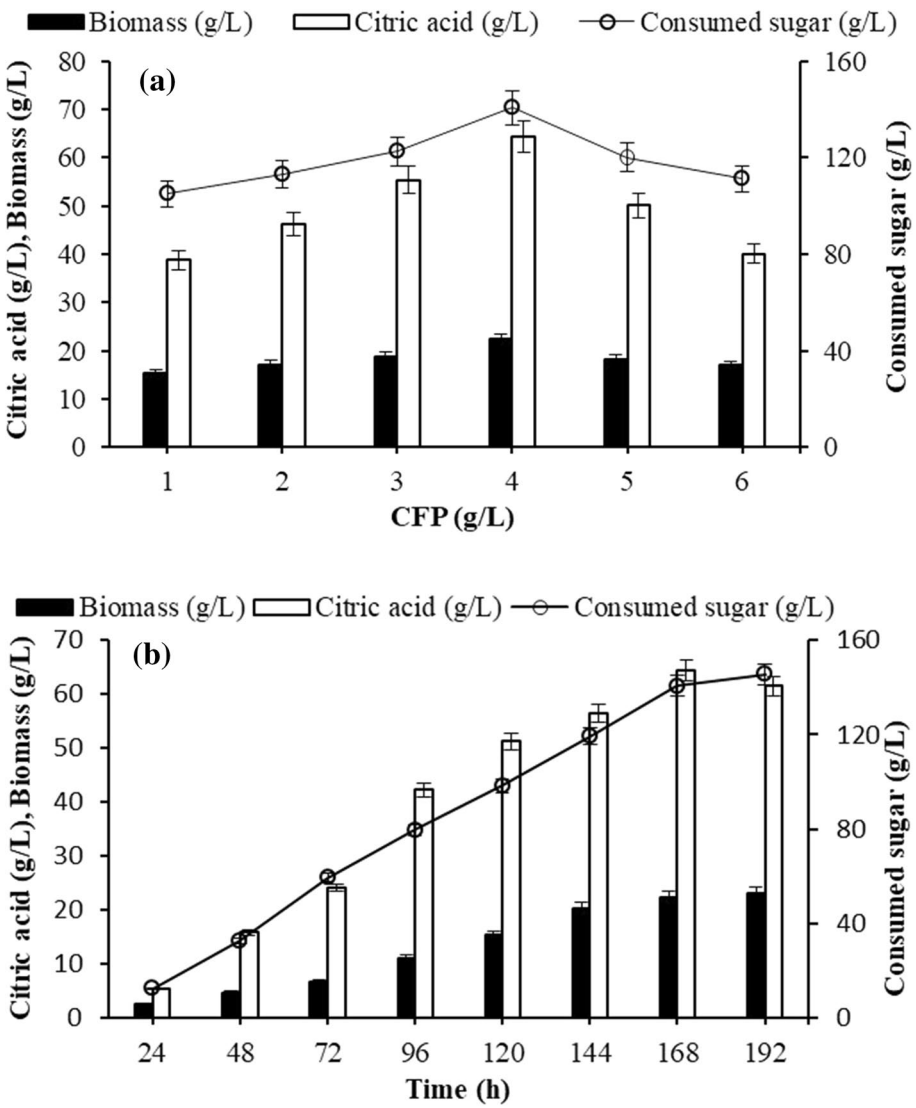

Fig. 2 **a** Effect of different concentrations of CFP on citric acid fermentation, **b** time course of citric acid production by *A. niger* EB-12 with molasses sugar (15%) and CFP (4 g/L) as the substrates

to be supplemented with a nitrogen source [19, 48]. This indicated that the nutrients in the CFP (4 g/L) were suitable for microbial growth and citric acid production.

The effect of fermentation time on citric acid production is presented in Fig. 2b. Citric acid production started after 24 h of fermentation. Citric acid production starts typically in the exponential growth phase and reaches maximum at late exponential or stationary phase [9]. This finding indicates that citric acid is a primary metabolite. The maximum of 64.40 g/L citric acid production was achieved at 168 h in CFPM. Similar, the maximum citric acid production in *A. niger* has been reported to occur on the 7th day of fermentation by the researchers [49, 50]. After this optimum fermentation time did not show any increase in citric acid production. It might be due to the reduced available nitrogen and carbon sources in the fermentation medium, age of fungi and inhibitors produced by fungi itself [18, 50].

Effect of Different Organic Nitrogen Sources on the Citric Acid Production

The effect of different peptones such as CFP, casein peptone (CP) and bacto peptone (BP) at the concentration of 4 g/L were investigated on citric acid fermentation by the mutant *A. niger* EB-12 (Fig. 3). It was found that the CFP gave significantly high citric acid production in comparison with the other peptones ($P < 0.05$). These commercial peptones have low ash content [34] and they are fairly expensive organic nitrogen sources compared with CFP. However, rich amino acid and salt content of CFP stimulated cell growth and citric acid biosynthesis in *A. niger* EB-12. This indicates that the macro- and micronutrients in the CFP suitable for microbial growth and citric acid production. The citric acid yields with CFP, CP and BP as nitrogen sources were 45.7, 30.1 and 34.6%, respectively. To reduce the production costs and enhance yield of citric acid, the low cost nitrogen sources

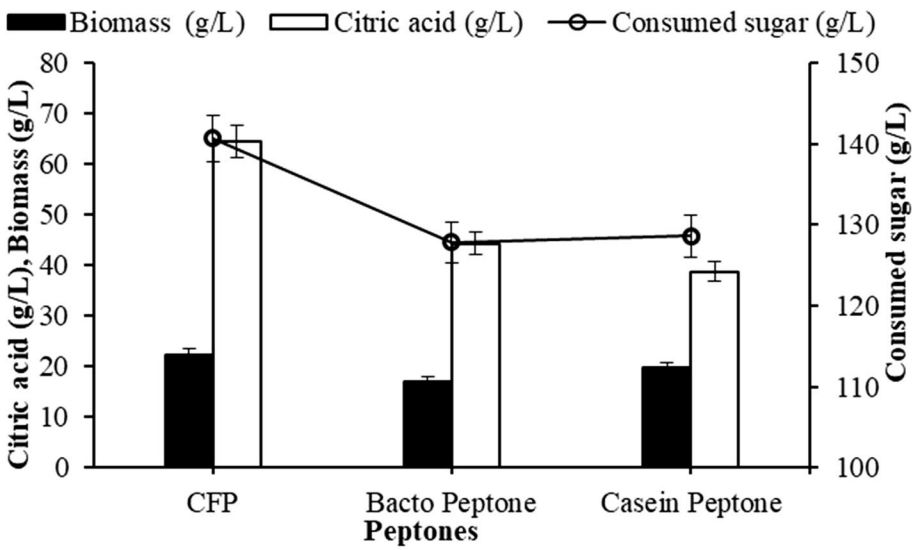

Fig. 3 Effect of different peptones sources on citric acid fermentation using molasses peptone medium. Culture conditions: ınitial pH 6.0, 200 rpm, 30 °C

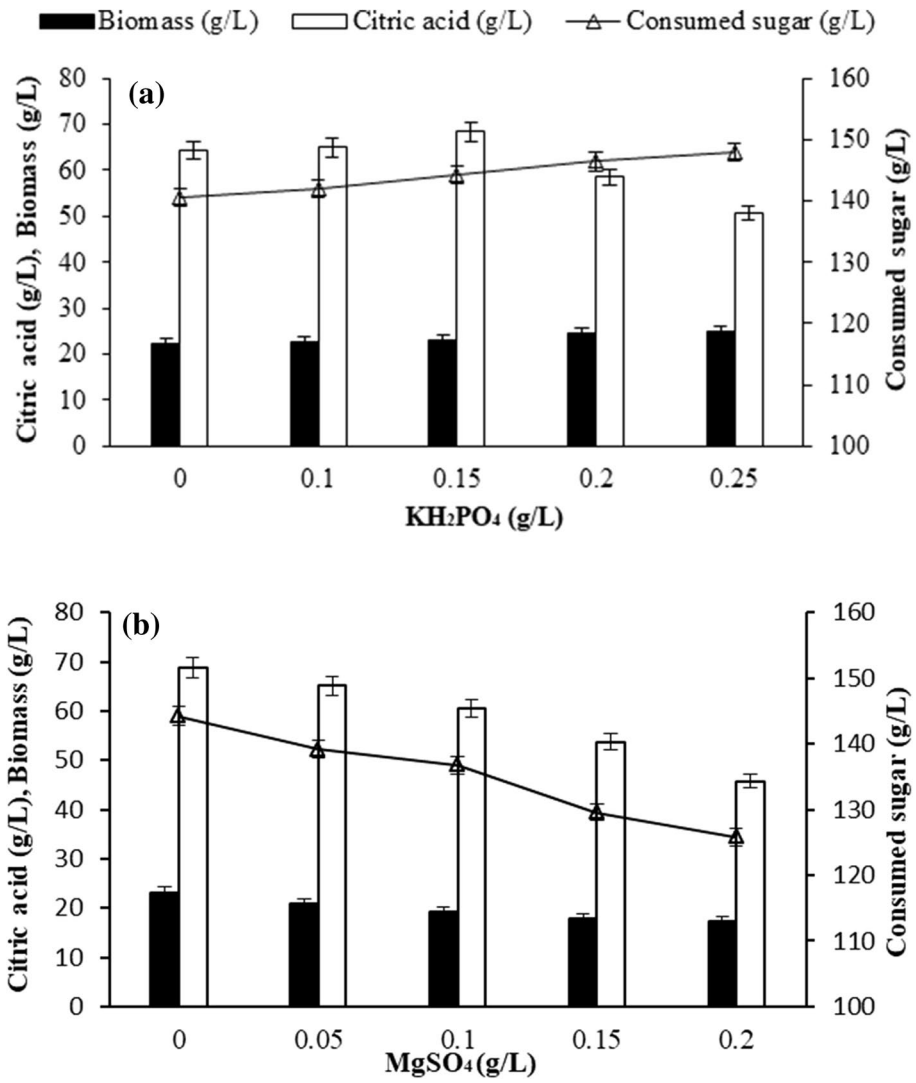

Fig. 4 Effect of KH_2PO_4 (**a**) and $MgSO_4$ (**b**) on citric acid production by mutant *A. niger* EB-12. Culture conditions: initial pH 6.0, 200 rpm, 30 °C, 168 h

Table 3 Citric acid production in different fermentation media containing sugar beet molasses

Citric acid (g/L)	Fermentation medium (g/L)	pH	Treatment	Cultivation conditions	References
113.50—74*	Sugar beet molasses (total sugar) 160, $NaNO_3$ 4, H_2PO_4 1, $MgSO_4{\cdot}7H_2O$, 0.23, $FeCl_3$ 0.02, $ZnSO_4$, 0.0012, $MnCl_2{\cdot}H_2O$ 0.0012	4	$CaPO_4$	F	[9]
87.81*	Sugar beet molasses (total sugar) 140, corn steep liquor 7.25, KH_2PO_4, 0.5, $MgSO_4{\cdot}7H_2O$ 0.15, $FeCl_3$ 0.01, $ZnSO_4$ 0.0006, $MnCl_2{\cdot}H_2O$ 0.0006	4	CaPO4	B	[51]
44.62—34*	Sugar beet molasses (total sugar) 150, $NaNO_3$ 4, KH_2PO_4 1, $MgSO_4{\cdot}7H_2O$ 0.23, $FeCl_3$ 0.02, $ZnSO_4$ 0.0012, $MnCl_2{\cdot}H_2O$ 0.0012, 1% ethanol	4	H_2SO_4	F	[53]
105—84*	Sugar beet molasses (total sugar) 150	5.8	$K_4Fe(CN)_6$	B	[48]
52.3—	Sugar beet molasses (total sugar) 140, $(NH_4)_2{\cdot}SO_4$ 2, KH_2PO_4 2, $MgSO_4{\cdot}7H_2O$ 0.5, $NH_4Fe(SO_4)_2{\cdot}12H_2O$ 0.9, $ZnSO_4{\cdot}7H_2O$ 0.5, $CuSO_4{\cdot}5H_2O$ 0.25	5	$K_4Fe(CN)_6$	F	[50]
68.80—48*	Sugar beet molasses (total sugar) 150, CFP 4, KH_2PO_4 0.15	6	$K_4Fe(CN)_6$	F	This study

*Yield

B bioreactor, *F* flask

such as ram horn hydrolyzate [24], corn steep liquor [51], and gelatin net hydrolysate [49] have been used as alternative nitrogen sources. These researchers reported that the ram horn hydrolyzate, gelatin net hydrolyzate and corn steep liquor increased citric acid yield 1.5, 2.5 and 14 times than the basal mediums, respectively. When CFP was used, it was determined that citric acid production increased 1.5 folds.

Effect of KH_2PO_4 and $MgSO_4$ on the Citric Acid Production

As shown in Fig. 4a, we investigated the effect of KH_2PO_4 on the production of citric acid. A range of KH_2PO_4 concentrations (0.1–0.25 g/L) were tested in order to identify the optimum KH_2PO_4 concentration for producing citric acid. As shown in Fig. 4a, the highest production rate of citric acid (68.8 g/L) was obtained by the addition of 0.15 g/L of KH_2PO_4 to the production medium. Low levels of phosphate have a positive effect on citric acid production [5, 52]. Berovic et al. [48] reported that production of citric acid (using sugar beet molasses) was achieved at only 50 mg/L H_3PO_4.

There was no need addition of MgSO4 as it was generated during the neutralization of the CFP (Fig. 4b). Further incease in the concentration of $MgSO_4$, gradually reduced citric acid synthesis, becoming low (45.78 g/L) at 0.2 g/L $MgSO_4$. As mentioned Table 1, CFP contains some of the magnesium salts such as $MgSO_4$ and $MgCl_2$, and consequently offers a rich source for culture media. The high concentration of Mg^{2+} inhibit citrate synthase which results in a decrease in the production of citric acid [37].

A wide range of citric acid production yield by *A. niger* using sugar beet molasses as carbon source has been reported. The citric acid production achieved in the present study with *A. niger* mutant EB-12 (68.8 g/L) using sugar beet molasses and CFP at optimal concentrations was much greater than the yields obtained in several previous studies (Table 3).

Conclusion

Agro-industrial wastes are increasing continuously due to the ever-increasing world population. The effective use of these residues for the production of bio-products by microorganisms has many advantages, such as value addition and waste management. The present study demonstrated the potential of molasses supplemented with CFP as alternative cheap substrate for citric acid production. The production of citric acid using different agro-industrial by-products with this strain is on-going in our lab. Bioreactor studies can be done to increase fermentation yield.

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References

1. Soccol, C.R., Vandenberghe, L.P., Rodrigues, C., Pandey, A.: New perspectives for citric acid production and application. Food Technol. Biotechnol. **44**(2), 141–149 (2006)
2. Prado, F.C., Vandenberghe, L.P.S., Woiciechowski, A.L., Rodrígues-León, J.A., Soccol, C.R.: Citric acid production by solid-state fermentation on a semi-pilot scale using different percentages of treated cassava bagasse. Brazilian J. Chem. Eng. **22**(4), 547–555 (2005)
3. Dhillon, G.S., Brar, S.K., Verma, M., Tyagi, R.D.: Recent advances in citric acid bio-production and recovery. Food Bioprocess Technol. **4**(4), 505–529 (2011)
4. Show, P.L., Oladele, K.O., Siew, Q.Y., Aziz Zakry, F.A., Lan, J.C.W., Ling, T.C.: Overview of citric acid production from *Aspergillus niger*. Front. Life Sci. **8**(3), 271–283 (2015)

5. Max, B., Salgado, J.M., Rodríguez, N., Cortés, S., Converti, A., Domínguez, J.M.: Biotechnological production of citric acid. Brazilian J. Microbiol. **41**(4), 862–875 (2010)
6. Wang, B., Chen, J., Li, H., Sun, F., Li, Y., Shi, G.: Pellet-dispersion strategy to simplify the seed cultivation of *Aspergillus niger* and optimize citric acid production. Bioprocess Biosyst. Eng. **40**(1), 45–53 (2017)
7. Zhou, P.P., Meng, J., Bao, J.: Fermentative production of high titer citric acid from corn stover feedstock after dry dilute acid pretreatment and biodetoxification. Bioresour. Technol. **224**, 563–572 (2017)
8. Gueguim-Kana, E.B., Oloke, J.K., Lateef, A., Oyebanji, E.: A.: Comparative evaluation of artificial neural network coupled genetic algorithm and response surface methodology for modeling and optimization of citric acid production by *Aspergillus niger* MCBN 297. Chem. Eng. Trans. **27**, 367–402 (2012)
9. Lotfy, W., Ghanem, K., Elhelow, E.: Citric acid production by a novel *Aspergillus niger* isolate: I. Mutagenesis and cost reduction studies. Bioresour. Technol. **98**(18), 3464–3469 (2007)
10. Rodrigues, C., de Souza Vandenberghe, L.P., Teodoro, J., Pandey, A., Soccol, C.R.: Improvement on citric acid production in solid-state fermentation by *Aspergillus niger* LPB BC mutant using citric pulp. Appl. Biochem. Biotechnol. **158**(1), 72–87 (2009)
11. Adeoye, A.O., Lateef, A., Gueguim-Kana, E.B.: Optimization of citric acid production using a mutant strain of *Aspergillus niger* on cassava peel substrate. Biocatal. Agric. Biotechnol. **4**(4), 568–574 (2015)
12. Javed, S., Asgher, M., Sheikh, M.A., Nawaz, H.: Strain improvement through uv and chemical mutagenesis for enhanced citric acid production in molasses-based solid state fermentation. Food Biotechnol. **24**(2), 165–179 (2010)
13. Ali, S., Iqbal, J.: Effect of volume of culture medium on enhanced citric acid productivity by a mutant culture of *Aspergillus niger* in stirred fermentor. Lett. Appl. Microbiol. **36**(5), 302–306 (2003)
14. Khurshid, S., Ali, S., Ashraf, H., Qadeer, M.A., Rajoka, M.I.: Mutation of *Aspergillus niger* for hyperproduction of citric acid from black strap molasses. World J. Microbiol Biotechnol. **17**(1), 35–37 (2001)
15. Wang, B., Li, H., Zhu, L., Tan, F., Li, Y., Zhang, L., Ding, Z., Shi, G.: High-efficient production of citric acid by *Aspergillus niger* from high concentration of substrate based on the staged-addition glucoamylase strategy. Bioprocess Biosyst. Eng. **40**(6), 891–899 (2017)
16. Kumar, D., Verma, R., Bhalla, T.C.: Citric acid production by *Aspergillus niger* van. Tieghem MTCC 281 using waste apple pomace as a substrate. J. Food Sci. Technol. **47**(4), 458–460 (2010)
17. Rodrigues, C., Vandenberghe, L.P., Sturm, W., Dergint, D.E., Spier, M.R., de Carvalho, J.C., Soccol, C.R.: Effect of forced aeration on citric acid production by *Aspergillus* sp. mutants in SSF. World J. Microbiol. Biotechnol. **29**(12), 2317–2324 (2013)
18. Dhillon, G.S., Brar, S.K., Kaur, S., Verma, M.: Screening of agro-industrial wastes for citric acid bioproduction by *Aspergillus niger* NRRL 2001 through solid state fermentation. J. Sci. Food Agric. **93**(7), 560–1567 (2013)
19. Nakata, H., Tamura, M., Shintani, T., Gomi, K.: Evaluation of baker's yeast strains exhibiting significant growth on Japanese beet molasses and compound analysis of the molasses types. J. Biosci Bioeng. **117**(6), 715–719 (2014)
20. Kamarudin, N.B., Sharma, S., Gupta, A., Kee, C.G., Chik, S.M.S.B.T., Gupta, R.: Statistical investigation of extraction parameters of keratin from chicken feather using Design-Expert. 3 Biotech **7**(2), 127 (2017)
21. Adelere, I.A., Lateef, A.: Keratinases: emerging trends in production and applications as novel multifunctional biocatalysts. Kuwait J. Sci. **43**(3) (2016)
22. Lateef, A., Oloke, J.K., Gueguim Kana, E.B., Sobowale, B.O., Ajao, S.O., Bello, B.Y.: Keratinolytic activities of a new feather-degrading isolate of *Bacillus cereus* LAU 08 isolated from Nigerian soil. Int. Bioremediat. Biodegradation **64**(2), 162–165 (2010). https://doi.org/10.1016/j.ibiod.2009.12.007
23. Lateef, A., Adelere, I.A., Gueguim-Kana, E.B.: Bacillus safensis LAU 13: a new source of keratinase and its multi-functional biocatalytic applications. Biotechnol. Biotechnol. Equip. **29**(1), 54–63 (2015). https://doi.org/10.1080/13102818.2014.986360
24. Kurbanoglu, E.B.: Enhancement of citric acid production with ram horn hydrolysate by *Aspergillus niger*. Bioresour. Technol. **92**(1), 97–101 (2004)
25. Kurbanoglu, E.B., Kurbanoglu, N.I.: Ram horn peptone as a source of citric acid production by *Aspergillus niger*, with a process. J. Ind. Microbiol. Biotechnol. **31**(6), 289–294 (2004)
26. Pitt, J.I., Hocking, A.D.: Fungi and food spoilage. Springer, New York (2009)
27. Magnani, M., Fernandes, T., Prete, C.E.C., Homechim, M., Ono, E.Y.S., Vilas-Boas, L.A., Sartori, D., Furlaneto, M.C., Fungaro, M.H.P.: Molecular identification of *Aspergillus* spp. isolated from coffee beans. Sci. Agric. **62**(1), 45–49 (2005)
28. A.O.A.C.: Official methods of analysis, 13th edn. Association of Official Agricultural Chemists, Washington, D.C. (1980)
29. Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L.: Rapid analysis of amino acids using pre-column derivatization. J. Chromatogr. B Biomed. Sci. Appl. **336**(1), 93–104 (1984)
30. Marier, J.R., Boulet, M.: Direct determination of citric acid in milk with an improved pyridine-acetic anhydride method. J. Dairy Sci. **41**(12), 1683–1692 (1958)
31. Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31**(3), 426–428 (1958)
32. Fuertes, J.B., Celada, J.D., Carral, J.M., Sáez-Royuela, M., González-Rodríguez, Á: Effects of fishmeal replacement by feather meal in practical diets for juvenile crayfish (*Pacifastacus leniusculus* Dana, Astacidae). Aquac. Nutr. **20**(1), 36–43 (2014)
33. Pan, L., Ma, X.K., Wang, H.L., Xu, X., Zeng, Z.K., Tian, Q.Y., Zhao, P.F., Zhang, S., Yang, Z.Y., Piao, X.S.: Enzymatic feather meal as an alternative animal protein source in diets for nursery pigs. Anim. Feed Sci. Technol. **212**, 112–121 (2016)
34. Taskin, M., Kurbanoglu, E.B.: Evaluation of waste chicken feathers as peptone source for bacterial growth. J. Appl. Microbiol. **111**(4), 826–834 (2011)
35. Ozdal, M., Gurkok, S., Ozdal, O.G.: Optimization of rhamnolipid production by *Pseudomonas aeruginosa* OG1 using waste frying oil and chicken feather peptone. 3 Biotech. **7**(2), 117 (2017)
36. Ozdal, M., Incekara, U., Polat, A., Gur, O., Kurbanoglu, E.B., Tasar, G.E.: Isolation of filamentous fungi associated with two common edible aquatic insects, *Hydrophilus piceus* and *Dytiscus marginalis*. J. Microbiol. Biotechnol. Food Sci. **2**(1), 95–105 (2012)
37. Ikram-ul, H., Ali, S., Qadeer, M.A., Iqbal, J.: Citric acid production by selected mutants of *Aspergillus niger* from cane molasses. Bioresour. Technol. **93**(2), 125–130 (2004)
38. Nagavalli, M., Ponamgi, S.P.D., Girijashankar, V., Rao, L.V.: Enhanced rifamycin SV production by submerged fermentation using *Amycolatopsis mediterranei*. Appl. Microbiol. Biotechnol. **99**(18), 7505–7513 (2015)
39. Koti, S., Govumoni, S.P., Gentela, J., Rao, L.V.: Enhanced bioethanol production from wheat straw hemicellulose by mutant strains of pentose fermenting organisms *Pichia stipitis* and *Candida shehatae*. Springerplus **5**(1), 1545 (2016)
40. Lesniak, W., Podgorski, W.: Effect of amino acids and vitamins on citric acid biosynthesis. Prog. Biotechnol. **17**, 251–256 (2000)
41. Ali, S.R., Anwar, Z., Irshad, M., Mukhtar, S., Warraich, N.T.: Bio-synthesis of citric acid from single and co-culture-based

fermentation technology using agro-wastes. J. Radiat. Res. Appl. Sci. **9**(1), 57–62 (2016)
42. Lal, D.N., Srivastava, A.S.: Effect of amino acids on microbial production of citric acid by *Aspergillus niger*. Zentralbl. Mikrobiol. **137**(1), 31–35 (1982)
43. Akram, M.: Citric acid cycle and role of its intermediates in metabolism. Cell Biochem. Biophys. **68**(3), 475–478 (2014)
44. Bari, M.N., Alam, M.Z., Muyibi, S.A., Jamal, P.: Improvement of production of citric acid from oil palm empty fruit bunches: optimization of media by statistical experimental designs. Bioresour. Technol. **100**, 3113–3120 (2009)
45. Pera, L.M., Callieri, D.A.: Influence of calcium on fungal growth and citric acid production during fermentation of a sugarcane molasses-based medium by a strain of *Aspergillus niger*. World J. Microbiol. Biotechnol. **15**(5), 647–649 (1999)
46. Haq, I.U., Ali, S., Qadeer, M.A., Iqbal, J.: Effect of copper ions on mould morphology and citric acid productivity by *Aspergillus niger* using molasses based media. Process Biochem. **37**(10), 1085–1090 (2002)
47. Kurbanoglu, E.B., Ozdal, M., Ozdal, O.G., Algur, O.F.: Enhanced production of prodigiosin by *Serratia marcescens* MO-1 using ram horn peptone. Brazilian J. Microbiol. **46**(2), 631–637 (2015)
48. Berovic, M., Rošelj, M., Wondra, M.: Possibilities of redox potential regulation in submerged citric acid bioprocessing on beet molasses substrate. Food Technol. Biotechnol. **38**(3), 193–201 (2000)
49. Lotfy, W.A.: Gelatin net hydrolyzate: a novel enhancer for citric acid production by *Aspergillus niger*. Res. J. Microbiol. **5**(11), 1131–1137 (2006)
50. Çevrimli, B.S., Yasar, A., Kariptas, E.: Effects of fermentation conditions on citric acid production from beet molasses by *Aspergillus niger*. Asian J. Chem. **21**(4), 3211–3218 (2009)
51. Lotfy, W., Ghanem, K., Elhelow, E.: Citric acid production by a novel *Aspergillus niger* isolate: II. Optimization of process parameters through statistical experimental designs. Bioresour. Technol. **98**(18), 3470–3477 (2007)
52. Mostafa, Y.S., Alamri, S.A.: Optimization of date syrup for enhancement of the production of citric acid using immobilized cells of *Aspergillus niger*. Saudi. J. Biol. Sci. **19**(2), 241–246 (2012)
53. Nadeem, A., Syed, Q., Baig, S., Irfan, H., Nadeem, M.: Enhanced production of citric acid by *Aspergillus niger* M-101 using lower alcohols. Turk. J. Biochem. **35**(1), 7–13 (2010)