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Citric Acid Production from Nontreated Beet Molasses by a Novel *Aspergillus niger* Strain: Effects of pH, Sugar and Ingredients

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Abstract

Effects of factors on the citric acid production from non-treated beet molasses were studied in *Aspergillus niger* OE55. Maximum amount of citric acid (19.13 and 34.62 g/L) was achieved when the initial pH of fermentation medium was 6.0 from 200 g/L and 150 sugar respectively. Citric acid production and biomass formation continuously increased during fermentation period in the media initially containing 200 g/L sugar. Remaining sugar (from 3.20 to 6.03 g/L) was higher at the end of fermentation in the media initially containing 160 g/L sugar than 200 g/L sugar. Yield of citric acid after 4 days of fermentation were ranged from 0.16 to 0.28 g/g from 160 g/L sugar. The high phosphorus and nitrogen levels stimulated biomass formation and reduced citric acid production. The optimum incubation for maximal citric acid production varies both with the sugar concentration in non-treated molasses and fermentation conditions for the novel *A. niger* strain. pH of fermentation media cannot have reduced below 4.72 and 3.35 in the media containing 200 and 160 g/L sugar, respectively, during fermentation, therefore citric acid production was not increased over 19.96 and 34.58 g/L respectively. This would be due to the formation of nitrogenous and polysaccharide compounds from molasses. Strain development in the citric acid production from wild strains depends mainly on the fermentation conditions.

Keywords: *Aspergillus niger*; Citric acid, Trace elements, Beet molasses

Introduction

Citric acid is widely used in the food, beverages, chemical, pharmaceutical, cosmetic and other industries [1]. It is mainly produced from submerged fermentation processes. Different *Aspergillus niger* strains have been used in the production of citric acid. The fermentation conditions optimization for mutated strain is primary importance in the development of any fermentation process owing to their impact on the economy [2,3]. Most of *A. niger* mutant strains, however, are not able to can produce commercially acceptable citric acid yields. *A. niger* can utilize cheap raw materials and produce higher amount of citric acid, thereby making the process more economical. The fermentation process involves the use of sugar (such as glucose and sucrose) supplemented with limited concentration of phosphate, nitrogen and trace elements. In all fermentation process, the use of carefully selected mutant strains is essential. For the strain to be viably used as industrial strain, it is screened for industrial citric acid production ability. Mutated strains have been found to produce more citric acid than the parent [4,5].

Citric acid production is strongly influenced by the composition of medium, especially concentration of carbon, phosphate, nitrogen and trace-metal ions. The presence of trace metals in toxic concentrations can be a significant problem during the fermentation of substrates into products. Concentrations of trace metals in molasses may be controlled by the treatment with ferrocyanide ion and treated molasses has been extensively used for citric acid production by *A. niger* [1,6]. The addition of potassium ferrocyanide enhanced the yield of citric acid. The sensitivity of the *Aspergillus* strains in their ability to produce citric acid requires treatment of beet molasses to remove ions (iron, zinc, copper, manganese and molybdenum) by means of precipitation, clarification and filtration, with or without active carbon. This treatment increases the cost of citric acid. Therefore, there is a requirement to *A. niger* strain able to produce citric acid from non-treated beet molasses. The citric acid concentration produced by wild strains is too low for economical processes, strain improvement was carried out to develop mutants of parent strain for increased production of the products [7,8]. However, strain development from wild strains to mutants depends mainly on the process of mutagenesis (physical and chemical agents). Developments of mutant strains which can synthesize higher concentration of citric acid within a short fermentation time and capable of growing at lower pH are preferred. The yield of citric acid was further enhanced by optimizing the fermentation parameters like temperature, pH, incubation time, substrate concentration, nitrogen source and several other ingredients to accumulate citric acid including strains of *A. niger* [7,9,10].

Turkey being an agricultural country is producing beet molasses more than 7.1 x 10^5 tons annually in 2014 and it is cheap raw material for citric acid production. Citric acid is one of the most useful organic acids in different industries and its worldwide demand is increasing day by day. So, the development of this technology would be highly beneficial. The present work, therefore, is concerned to improve a novel *A. niger* mutant strain for production of citric acid by submerged fermentation process using non-treated beet molasses. For this aim, optimization of fermentation parameters was studied: sucrose concentration, initial pH, phosphate, ammonium and trace elements.

Materials and Methods

Microorganism and beet molasses

The mold used in this study is a UV-mutant of a novel *A. niger* strain isolated from a beed mud sample (Erkmen, 2017) and is deposited in the Department of Food Engineering (microbial culture collection), University of Gaziantep (27310 Gaziantep, Turkey) with the strain code OE55. Beet molasses used in this research was obtained from Kayseri Sugar Ltd. (Kayseri, Turkey).

Batch fermentation

Flow sheet of citric acid fermentation process by *Asepergillus niger* is given in Figure 1. Spores suspension of *A. niger* OE55 was prepared by washing 3 days old culture potato dextrose agar (Difco) plate with sterile saline solution (0.9% NaCl) and shaking vigorously for 1 min. Spores were counted by a haemocytometer to adjust the count to approximately 10^6 spores/ml. The inoculum medium consisted of 120 g/l sugar in non-treated beet molasses, 2.5 g/l NH_4NO_3, 0.16 g/l KH_2PO_4, 0.25 g/l $MgSO_47H_2O$, 1.0 ppm Zn ($ZnSO_47H_2O$), 1.0 ppm Fe ($Fe_2SO_424H_2O$), 1.0 ppm Cu ($CuSO_45H_2O$) and pH 6.0. The inoculum culture for fermentation media was grown in shake flasks for 24 h at 30°C and 220 rpm in a shaker (NUVE ST-402, İstanbul, Turkey).

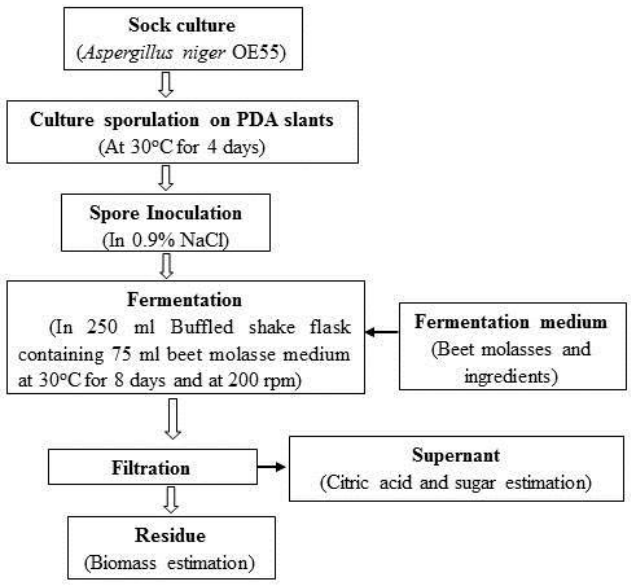

Figure 1: Flow sheet of citric acid fermentation process by *Asepergillus niger*.

The fermentation media were prepared from non-treated beet molasses together with different ingredients as indicated in Table and used in shake flask studies [5,10]. pH of the fermentation media is adjusted to 6.0. The fermentation with P2 media were also made at pH 4.0 and 2.5. Sixty-five mL fermentation medium was dispensed into 250 mL Erlenmeyer flasks and sterilized. Ten mL inoculum culture were used to inoculate the fermentation flasks which were then incubated at 30 ± 1°C under shaking conditions at 220 rpm in the shaker for 8 days. About 10 ml of samples (cultures) were removed at each sampling time (0, 2, 4, 6 and 8 days of fermentation) under aseptic conditions and used in analysis.

Analysis

Ten ml sample was filtered through a pre-weighed Whattman filter paper No. 44. The filtrate was used for further analysis (citric acid, sugar and pH). Filter paper was washed with distilled water and dried in an oven (Model: 1442 A, Memmert, Germany) at 105°C for overnight to calculate the biomass [5,10].

Determination of citric acid and sugar concentrations

The filtrate was used in the analyzes of pH, citric acid and sugar. pH was detected using a pH meter equipped with an electrode. Citric acid and sugar were determined by HPLC instrument equipped with a UV and refractive index detector (RID) respectively. Nucleosil column (EC 4.6 x 250 mm) and Shodex Sugar SH1011 column (8 x 300 mm, Bio-Rad, USA) were used for citric acid and sugar analysis, respectively, [5,9,10]. The eluent used for both citric acid and sugar analysis was 5 mM H_2SO_4 (aq.). HPLC analyses were carried out under the following operation conditions: pump flow, 1.0 ml/min; column temperature, 30°C for citric acid and 60°C for sugar; sample amount, 20 µl; and integration method, peak area. Concentrations were calculated from standard curve. Sugar concentration was calculated as the sum of sucrose, glucose and fructose concentrations. The yield of product from substrate (YPS) calculated by formula: YPS=(g citric acid /g sugar consumed).

Sample analyses were run parallel in duplicate. Each treatment was repeated three times and the results were reported as average of three repetitions.

Statistical analysis

All the statistical analyses were performed using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). The significance was set to $p<0.05$ for the ANOVA matrix F value among treatments using one-way and multiple-range analysis of variance to compare means on the assays.

Results and Discussion

A. niger is very sensitive to trace metal ions in the beet molasses. Despite treatment with chelating agents (such as sodium or potassium ferro-cyanide), molasses give low yields of citric acid [1,2]. Therefore, beet molasses requires treatment before use in the production of citric acid. There is a requirement to find the mutant *A. niger* strain able to produce citric acid from non-treated beet molasses. In this study, a novel *A. niger* OE55 mutant strain has been tested to able to produce citric acid from non-treated beet molasses under different fermentation media (Table 1). Since, this type of strain necessary to reduce cost of citric acid production.

Three initial pH (6.0, 4.0 and 2.5) for P1 medium with 160 g/L sugar was used to indicate the effect of pH on the citric acid production by *A. niger*. Increasing the initial pH increased citric acid production. When the initial pH was decreased to 4.0 or 2.5 from 6.0, the production of citric acid decreased (data not given). The favorable initial pH is very essential for the successful production of citric acid. The results showed that low pH of non-treated beet-molasses was inhibitory on the production of citric acid by *A. niger*. At low initial pH, the metalic ions present in non-treated molasses may be more toxic on the morphology

of mold and production of citric acid. This finding is an agreement with the observations of Pessoa et al. [11,12] and Haq et al. [12].

Medium Type	Ingradients (g/L)*						
	Mg	N	P	Zn	Fe	Mn	Cu
P1	-	-	-	-	-	-	-
P2	0.5	1.0	1.0	10^{-6}		-	10^{-6}
P3	0.5	0.5	1.5	10^{-4}	10^{-3}	10^{-6}	10^{-5}
P4	0.5	1	1.0	10^{-6}	10^{-5}	10^{-6}	10^{-6}
P5	0.5	1.5	2.0	10^{-6}	10^{-5}	10^{-5}	-
P6	0.2	1.5	2.0	10^{-6}	10^{-4}	-	-
P7	0.2	1.0	1.0	10^{-6}	10^{-4}	10^{-6}	-
P8	0.5	1.5	1.0	10^{-6}	10^{-6}	-	10^{-5}

Table 1: Ingradients added into nontretaed beet molasses media containing 200 and 160 g/L sugar. *Mg=$MgSO_47H_2O$, N=$(NH_4)_2SO_4$, P=KH_2PO_4, Zn=$ZnSO_47H_2O$, Fe=$Fe_2SO_424H_2O$, Mn=$MnSO_47H_2O$, Cu= $CuSO_45H_2O$.

A higher initial pH during fermentation process can also lead to the accumulation of other organic acids [12]. The initial pH of the medium is important in two stages of the fermentation process. Citric acid fermentation starts from spores and their germination requires pH>5.0. The absorption of ammonia by germinating spores causes release of protons, thus lowering the pH (near 2.5) and improving the production of citric acid [12-14]. But the pH of fermentation media was not reduced below 4.72 and 3.35 in the media containing 200 and 160 g/L sugar, respectively, during fermentation, therefore citric acid production was not increased over 19.96 and 34.58 g/L respectively. This would be due to the formation of nitrogenous and polysaccharide compounds from molasses. In this research, pH of fermentation media containing 160 g/L sugar were increased after 4 days. The nitrogenous compounds in molasses, formation of nitrogenous and polysaccharide compounds, and hydrolysis of mold cells be caused pH increase. Inactivation of a citrate degrading enzyme (e.g. aconitase or isocitrate dehydrogenases) essential for the accumulation of citric acid [10]. Extending the fermentation beyond 4 days in the case of 160 g/L sugar use would also be resulted in oxidation of citric acid by *A. niger*. Since most of the fermentable sugar utilized by *A. niger* within 4 days of fermentation. It has been reported [15,16] that *A. niger* oxidized the accumulated citric acid upon exhaustion of the fermentable sugar. Lower amounts of citric acid were produced from 200 g/L sugar than 160 g/L sugar. Therefore, CA utilization cannot be occurred and the increase in pH was not observed.

The effect of two sugar concentrations (160 and 200 g/L) on the citric acid production by *A. niger* were carried out at initial pH 6.0 (Table 2). Reduction in citric acid formation was observed when the sugar concentration of molasses increased from 160 g/L sugar to 200 g/L. The maximum amount of citric acid (19.00 g/L) was obtained in the P4 medium containing 200 g/L sugar while citric acid productions were ranged from 21.82 to 34.69 g/L after 4 days of fermentation in the media containing 160 g/L sugar. The citric acid productions were slightly increased in fermentation media containing 200 g/L sugar. Remaining sugar (from 3.09 to 6.13 g/L) was higher after 8 days of fermentation in the molasses initially containing 160 g/L sugar than the molasses initially contains 200 g/L sugar. The biomass formation were ranged from 28.83 to 58.50 and 11.20 to 26.70 g/L with 200 and 160 g/L sugar, respectively, after 8 days of fermentation period. Low amount of citric acid production may be due to the over growth of the mycelium, which resulted in increased viscosity of the medium (Table 1) and higher amount of trace elements in beet molasses (200 g/L sugar) than dilute (160 g/L sugar). Since biomass formations were higher (P>0.05) in the media containing 200 g/L sugar than 160 g/L sugar. Similar results were also observed by Haq et al. [12], they indicated that there was reduction in the production of citric acid with the increase of mycelial formation in the medium. Yield of citric acid after 4 days of fermentation were ranged from 0.16 to 0.28. The higher yield (0.1 g/g) of product was obtained in the P4 medium after 8 days of fermentation with 200 g/L sugar while it was 0.28 g/g in the P2 media after 4 days of fermentation with 160 g/L sugar.

Medium type	Days	Citric acid fermentation by 200 g/L sugar					Citric acid fermentation by 160 g/L sugar				
		CA (g/L)	Yield (g/g)	Remainin sugar (g/L)	pH	Biomass (g/L)	CA (g/L)	Yield (g/g)	Remaining Sugar (g/L)	pH	Biomass (g/L)
P1	2	0.74	0.03	29,31	4,72	10,33	5,02	0.16	31,75	3,88	4,16
	4	1.93		12,96	4,98	18,60	20,20		26,87	4,11	9,48
	6	3.39		5,25	4,66	25,07	17,90		12,23	4,42	10,62
	8	4.95		1,78	4,64	28,83	8,49		3,74	4,94	12,10
P2	2	1.35	0.08	23,59	4,88	19,13	6,59	0.28	85,48	3,35	7,76
	4	3.31		11,60	4,78	30,38	34,69		34,20	3,86	9,90
	6	7.51		7,99	4,76	47,04	22,37		14,67	5,43	12,61
	8	15.36		2,03	4,76	53,75	15,20		5,76	5,81	17,42
P3	2	1.28	0.03	39,08	5,13	14,92	5,80	0.2	78,13	3,89	12,20
	4	2.07		13,06	4,51	29,72	24,20		31,75	4,33	16,92

	6	3.31		8.21	4,48	42,92	23,89		12,61	4,76	17.3
	8	6,72		2.08	4,46	49,20	21,27		3,20	4,94	21.69
P4	2	1.41	0.1	37.85	5,57	17,26	22,75	0.25	56,16	3,87	8,62
	4	1.94		17.71	5,55	28,57	31,58		23,21	3,96	10,12
	6	12,75		8.5	4,89	39,08	23,89		8,18	4,00	11,18
	8	19.56		2.04	4,75	46,76	6,85		4,88	4,20	26,44
P5	2	3.67	0.07	22.02	5,46	18,2	17,33	0.17	28,19	3,81	12.31
	4	7.74		11.77	5,30	32,43	21,82		19,55	3,93	15.18
	6	11		6.2	5,08	53,93	25,20		16,87	4,60	19.98
	8	13.47		1.12	5,02	68,35	20,54		3,09	5,04	20.4
P6	2	2.36	0.07	39.59	5,39	16,52	14,71	0.16	42,10	3,88	10,3
	4	5.46		18.5	5,08	39,27	23,20		26,66	4,11	12,70
	6	9.03		9.21	4,98	54,62	20		6,37	4,42	15,75
	8	13.59		2.05	4,83	58,50	4,49		5.64	4,94	22,45
P7	2	2.34	0.09	28.827	5,86	14,20	23,06	0.27	39,31	3,88	11.61
	4	5.2		12.7	5,47	31,78	33,89		25,65	4,11	19.38
	6	12.78		7.36	5,27	36,40	7,90		9,789	4,42	25.8
	8	17.3		1.25	5,17	49.83	4,75		4.52	4,94	26.7
P8	2	4,11	0.07	32.61	5,63	20,59	7,61	0.23	31,75	3,88	14.78
	4	9		17.32	5,40	33,63	27,89		26,87	4,11	20.37
	6	11.77		10.2	5,40	64,40	20,20		19,55	4,42	23.44
	8	13.13		2.09	5,32	52,47	19.85		6,13	4,94	25.16

Table 2: Citric acid (CA) production, yield, pH and biomass formation from untreated beet molasses.

In batch-wise fermentation of citric acid, the production starts after a lag phase of one day and reached maximum at the onset of stationary phase or late exponential phase [6]. In the case of 160 g/L, maximum citric acid formation reached after 4 days of fermentation. Since, most of sugar has been consumed within 4 days in the fermentation media. Our finding is not more economical as compared to previous research [7-9,13]. This would be due to the use of non-treated molasses in the preperation of fermentation media. Biomass formations in this research from 200 and 160 g/L sugar were higher than the results of these research in literature.

Nitrogen was provided in the beet molasses media as ammonium nitrate ($(NH_4)_2SO_4$). Citric acid production is directly influenced by the concentration of the nitrogen. Ammonium compound consumption leads to pH decrease, which is essential for the citric fermentation. It is necessary to maintain initially high pH values in the first day of fermentation prior to a certain quantity biomass production [10]. The results showed that initial concentration of nitrogen source required for citric acid fermentation is 1.0 g/L for the non-treated molasses (Tables 1 and 2). High nitrogen concentration (1.5 or 3.3 g/L) increased mold growth (P5, P6 and P7) and sugar consumption but decrease the citric acid produced.

Phosphorous was provided in the beet molasses media as di-potassium phosphate (KH_2PO_4). Tables 1 and 2 show that 1.0 g/L KH_2PO_4 in the fermentation medium was an optimum concentration as phosphorus source in the production of citric acid. A low and high phosphorus caused a drastic reduction of citric acid production. This was reflected in the lower yield of citric acid, which were severely reduced as the phosphate levels increased (P5, P6 and P7) or decreased (P3). Again, the high phosphorus level stimulated biomass formation.

Citric acid production and yields can be significantly ($p>0.05$) increased with using Mg^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and Cu^{2+} ions in ppm, above which the process is negatively affected (Tables 1 and 2). For instance, iron, one of the cofactors of aconitase, plays a crucial role, favoring biomass growth at concentrations higher than 2 ppm. Cu reverses the effect of Fe [13,17]. Biomass formation continuously increased during fermentation. The addition of nitrogen, magnesium and phosphate sources further increased the production of citric acid. The mutant strain of *A. niger* OE55 supported maximum production of citric acid (34.69 g/l) from nontreated beet molasses. The ions that should be in limiting concentrations are heavy metals. The results also showed that the necessity to use treated beet molasses medium to produce higher amount of citric acid by tested mutated *A. niger* OE55

strain. The optimum time of incubation for maximal citric acid production varies both with the sugar concentration in non-treated molasses.

Conclusion

The present results indicate that there should be an optimum carbon, nitrogen, phosphate and magnesium concentration in the fermentation medium and any excess amount reduced citric acid formation, while they change the direction of fermentation to biomass over production. The research showed that mutant strain is very sensitive to trace metal ions in the beet molasses. Despite treatment with chelating agents (such as sodium or potassium ferro-cyanide), molasses give low yields of citric acid. Therefore, this strain must be tested using treated beet molasses in the production of citric acid. There is a requirement to find the mutant *A. niger* strain able to produce citric acid from non-treated beet molasses.

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