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Enhanced Production of Citric Acid by *Aspergillus niger* M-101 Using Lower Alcohols

[*Aspergillus niger* M-101 Tarafından Üretilen Sitrik Asit Miktarı Düşük Molekül Ağırlıklı Alkoller Kullanıldığında Artmaktadır]

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ABSTRACT

Objective: Citric acid is mainly produced by submerged fermentation of *Aspergillus niger*. The process yield depends on the composition of the medium, as well as on the microorganism strain.

Materials and Methods: In the present study citric acid production by *Aspergillus niger* M-101 using lower alcohols such as methanol and ethanol at concentration of 0.5 to 2.0 % (v/v) was optimized in shake flask culture using beet molasses as fermentation medium.

Results: Methanol (1.5 %) and ethanol (1 %) was found optimal for maximum citric acid production (49.33±4.23 g/l) and (40.85±0.48 g/l) respectively. The effect of addition of ethanol and methanol to the fermentation medium at different time intervals on citric acid production demonstrate that maximum citric (44.62±2.12 g/l) and (55.13±1.53 g/l) was produced after 30 h and 45 h of incubation period. The yield for methanol and ethanol was 1.54 and 2.33 higher than the control experiment.

Conclusion: From the present study it is concluded that both methanol and ethanol under optimized conditions increases the permeability of cell membrane of *Aspergillus niger* M-101 that result in accumulation of citric acid in fermentation medium. The kinetic assessments demonstrate that there was a significant enhancement ($p \leq 0.05$) in citric acid production over the control fermentation medium. The best results for the production of citric acid were observed after 192 h of fermentation period.

Key Words: *Aspergillus niger*, methanol, ethanol, citric acid, kinetic assessments

ÖZET

Amaç: Sitrik asit *Asregillus niger* tarafından batık kültür fermentasyonu ile üretilmektedir. Bu üretimin verimi mikroorganizmanın suşuna bağlı olduğu kadar besi ortamının bileşiminden de etkilenmedir.

Methods: Çalışmada *Aspergillus niger* M-101 tarafından sitrik asit üretimi, % 0.5-2 derişimde methanol ve etanol gibi düşük molekül ağırlıklı alkollerin varlığında sallamalı kültür ortamında optimize edildi. Fermentasyon ortamı olarak pancar melası kullanıldı.

Bulgular: Metanol (% 1.5) ve etanol (% 1) fazla miktarda sitrik asit üretimi için (sırasıyla 49.33±4.23 g/l ve 40.85±0.48 g/l) uygun olarak saptandı. Farklı zaman aralıklarında fermentasyon ortamına alkol eklenmesinin sitrik asit üretimine olan etkisine bakıldığında; etanol için 30 saat, methanol için ise 45 saat inkübasyon süresinden sonra en fazla üretimin (sırasıyla 44.62±2.12 g/l ve 55.13±1.53 g/l) gerçekleştiği bulundu. Kontrol grubuna göre kıyaslandığında methanol ve etanol eklendiğindeki verim 1.54 ve 2.33 olarak hesaplandı.

Tartışma: Optimize edilmiş koşullar altında methanol ve etanol *Aspergillus niger* M-101'in hücre membranı geçirgenliğini arttırmakta ve sitrik asit fermentasyon ortamında birikmektedir. Kinetik çalışmalar kontrol grubuna kıyasla sitrik asit üretiminde belirgin bir artış oluğunu ($p \leq 0.05$) göstermektedir. Sitrik asit üretimi için en iyi sonuçlar 192 saatlik fermentasyon döneminden sonra elde edilmektedir.

Anahtar Kelimeler: *Aspergillus niger*, metanol, etanol, sitrik asit, kinetik değerlendirme

Introduction

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) is a tricarboxylic acid with a molecular weight of 210.14 Da. In view of its three carboxylic acid functional groups, it has three pKa values at pH 3.1, 4.7, and 6.4. Citric acid is a nearly universal intermediate product of metabolism and its traces are found in virtually all plants and animals (1). It is solid at room temperature, melts at 153 °C and decomposes at higher temperature (2).

Among the organic acids industrially produced, citric acid is the most important in quantitative terms with an estimated annual production of about 1.4 million tons (3). The annual growth of its demand/consumption rate is around 3.5-4.0 % (4). The food industry consumes about 70 % of total citric acid produced and pharmaceutical industries consume about 12 %, and the remaining 18 % are consumed by other industries (4-5). Citric acid is used in the food, beverage, pharmaceutical, chemical, cosmetic and other industries for applications such as acidulation, antioxidation, flavour enhancement, preservation, plasticizer and as a synergistic agent (6). Citric acid is used to flavour the drinks, jams and jellies, candies, water ice and wines (7).

Citric acid production can be done by solid-state fermentation or with submerged fermentation (8). To date, most industrial processes are carried out with submerged fermentation of *Aspergillus niger*. The process yield depends on the composition of the medium, as well as on the microorganism strain (9). *Aspergillus niger* has been used commercially for the first time in 1923 for citric acid production and it remained the organism of choice for commercial production because it produces more citric acid per time unit. Recently a wide range of citric acid production has been reported in response to different levels of nutrient supplementation (10-13).The problem in the production of citric acid for yeasts is the simultaneous formation of isocitrate. The main advantages of using *Aspergillus niger* are its ease of handling, its ability to ferment a variety of cheap raw materials and high yields (3,14). The yield of citric acid from these strains often exceeds 70 % of the theoretical yield on the carbon source (1). The production of citric acid using cheap carbon source from agro-industrial byproducts provides considerable combined benefit of waste material management as well as decrease of citric acid production cost (15-16).

The use of alcohols as a stimulant in citric acid production may enhance the yield of citric acid (17). The stimulatory effect of ethanol and methanol on citric acid production can be explained in terms of mycelia morphology as well as pellet shape and size (18). Citric acid production could be increased by exploiting available resources and adding the stimulatory agents to the fermentation medium (19). The main purpose of the present study was to investigate the stimulatory effect of lower alcohols on citric acid production and its optimization.

Materials and Methods

Microorganism

Aspergillus niger M-101 was obtained from Pakistan Type Culture collection at Food Biotechnology Research Centre PCSIR Laboratories, Lahore, Pakistan. It was maintained on Potato dextrose agar (PDA) slants and stored at 4 ºC in a refrigerator. The culture was renewed after every month throughout the investigation period.

Composition of beet molasses

Approximate analysis of beet molasses was done according to the method of Ranganna (20). Molasses (obtained from Premier Sugar Mills, Mardan, NWFP, Pakistan) used in the experimental work was initially homogenized. Suitable dilution of molasses was done as required by the experiments. Composition of molasses used in this study is given in Table 1.

Table 1. Proximate analysis of beet molasses

Constituent	Dry weight basis (%)
Moisture	19.4
Dry Solids	81.6
Ash	8.5
Total Reducing Sugar	17.21
Total Sugar	60.19
Sucrose (non reducing sugar)	42.98
Nitrogen	0.38

Clarification of beet molasses

Clarification of beet molasses using sulfuric acid treatment was done according to the method of Mayilvahanan et al. (21). The pH of the molasses (25 % total sugar) was adjusted to 3.0 by adding 0.1 N sulphuric acid. This was allowed to stand for 1.5 hours and then centrifuged at 3000 rpm for 15 minutes. Clarified beet molasses was diluted with distilled water to obtain 150 g/l sugar concentration. The supernatant was collected and used for citric acid production.

Basal fermentation media

The following fermentation media (g/l) was employed for citric acid production according to the modified method of Lotfy et al. (22): Beet molasses, 150.0; $NaNO_3$, 4.0; KH_2PO_4, 1.0; $MgSO_4$ $7H_2O$, 0.23; $FeCl_3$, 0.02; $ZnSO_4$, 0.0012; $MnCl_2 \cdot H_2O$, 0.0012 (pH 4±0.2).

Inoculum

The spore suspensions of fungal strains were prepared by washing 5-7 days old culture slants with sterilized saline solution (0.9 % NaCl) with shaking vigorously for 1min. Spores were counted by a haemocytometer to adjust the count to approximately 10^8 spores/ml.

Fermentation

The spores of *Aspergillus niger* M-101 was allowed to grow in 50 ml aliquots of the fermentation medium dispensed in 250 ml Erlenmeyer flasks. Each flask was inoculated with 1 % (v/v) inoculum containing 10^8 spores/ml. Cultures were then incubated at 30±2 °C under shaking conditions at 200 rpm for 192 h. The flow sheet of fermentation process is given in figure 1.

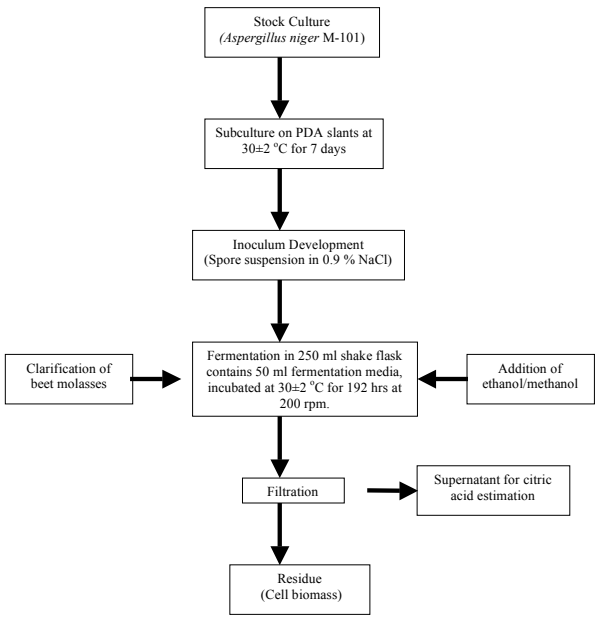


Figure 1. Flow sheet of fermentation process for the production of citric acid by Aspergillus niger M-101.

Analytical methods

The fermentation broth was filtered through filter paper in order to remove mycelia. The filtrate was then used for further analysis. Biomass was determined according to the method reported by Kiramura et al. (23). Citric acid in filtrate was estimated spectrophotometrically, using pyridine–acetic anhydride method as given by Marrier and Boulet (24). Reducing sugar was estimated by 3,5-dinitrosalicyclic acid (DNS) method (25).

Kinetic parameters and statistical analysis

Kinetic parameters for batch fermentation were determined after Pirt (26). Statistical analysis was done by ANOVA test using Minitab (version 15) software. The difference in values was indicated in the form of probability ($p \leq 0.05$) values.

Results and Discussion

Stimulatory effect of two different alcohols (methanol and ethanol) on citric acid production was studied. The date in Table 1 shows the proximate analysis of beet molasses. Moisture content, dry solids and ash content in beet molasses were 81.6, 19.4 and 9.0 % respectively, while amount of total reducing sugar and total sugars were 17.23 and 60.16 % respectively. While the amount of nitrogen present in beet molasses was 0.38 %. The reported values of molasses were also given by Pazouki et al. (27)

Citric acid production was increased gradually during the fermentation period and reached to its maximum value (27.25±1.35 g/l) after 8 days (Figure 2). At optimum fermentation period, biomass and sugar consumed were 32.2±1.71 g/l and 132.5±2.29 g/l, respectively. Increasing fermentation period did not improve citric acid production. This decrease in productivity might be due to inhibitory effect of high concentration of citric acid, decay in enzyme system responsible for biosynthesis of citric acid, and reduce the amount of nitrogen available in fermentation medium and depletion of sugar contents as reported by investigators (28-31). These findings were also in agreement to Lotfy et al. (22) and Shamrai and Orlaw (32). They reported that maximum productivity of citric acid was obtained after 8 days of fermentation period.

The effect of different concentration of ethanol and methanol (0.5-2.0 %) was presented in Figure 3 and 4. The maximum amount of citric acid (40.85±0.48) and (49.33±4.23 g/l) was produced when ethanol (1.0 %) and methanol (1.5 %) was added into the beet molasses medium while maximum percentage yield for on the basis of sugar consumed for citric acid was 33.07 % and 46.89 % respectively. According to our study both ethanol and methanol showed stimulatory effect on citric acid production. Our findings are in agreement to those reported by investigators (33,34). According to them lower concentration of ethanol and methanol has stimulatory effect on citric acid production. However increase in concentration of ethanol and methanol tends to decrease its productivity. This might be due to the fact that the higher ethanol and methanol concentration in the medium disturbed the fungal metabolism and inoculum morphology, which resulted in decrease citric acid production (33).

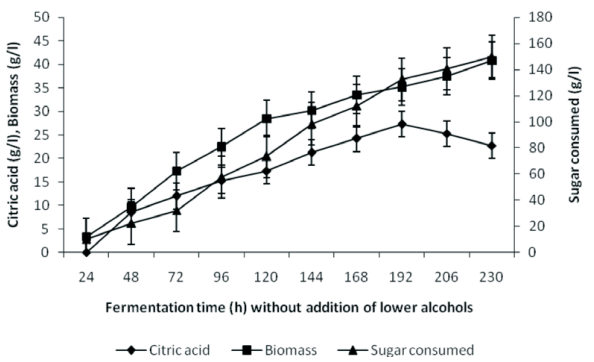
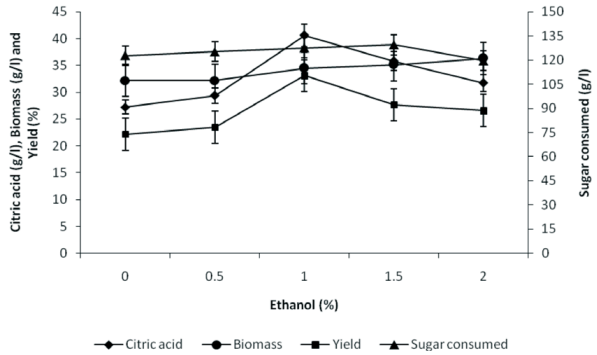


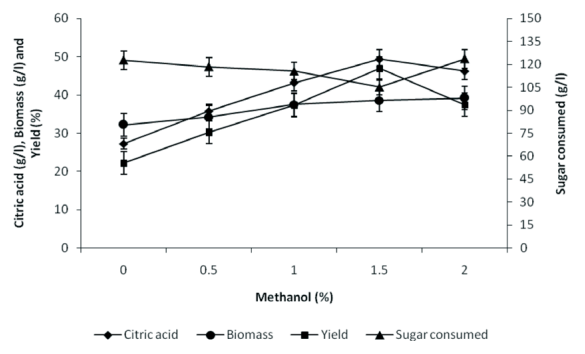
Figure 2. Effect of fermentation period on citric acid production
Effect of fermentation period on citric acid production revealed that the productivity of citric acid was tend to increase with increase in fermentation period and reached to its maximum value after 192 h of fermentation period but a sudden decrease in citric acid productivity was observed with further increase in fermentation period. Thus 192 h fermentation period was found optimum for citric acid production.



Yield (%) = (grams of citric acid produced/grams sugar consumed) x 100

Figure 3. Effect of different concentration of ethanol on citric acid production

Effect of different ethanol concentration (0.5-2.0 %) on citric acid production under optimal fermentation condition was studied. 1.0 % ethanol concentration was found to be optimal for citric acid production as maximum amount of citric acid was produced at this concentration, with further increase in ethanol concentration decrease in citric acid production occurred. All values shown in this figure differs significantly at $p \leq 0.05$.



Yield (%) = (grams of citric acid produced/grams sugar consumed) x 100

Figure 4. Effect of different concentration of methanol on citric acid production

Effect of different methanol concentration (0.5-2.0 %) on citric acid production under optimal fermentation condition was studied. 1.5 % methanol concentration was found to be optimal for citric acid production as maximum amount of citric acid was produced at this concentration, with further increase in methanol concentration decrease in citric acid production occurred. All values shown in this figure differs significantly at $p \leq 0.05$.

By comparing the effect of both ethanol and methanol on biomass growth it was observed that methanol has fruitful effect on biomass growth but there is no stimulatory effect of ethanol on biomass, which means that ethanol was solely used by *Aspergillus niger* as a carbon source for citric acid production. Similar findings were also reported by Barrington and Kim (18). The stimulating effect of ethanol on citric acid production suggests that ethanol could be used as a carbon source to be converted into citric acid via the TCA cycle of *A. niger* or it also increases the permeability of the cell membrane and, thus the secretion of citric acid (34-37). Methanol markedly depressed the synthesis of cell protein in the early stages of the cultivation (38) and also increased the metabolic activity of the enzyme citrate synthase (18). One of the effects of ethanol and methanol is to increase greatly the tolerance levels of manganese, iron, and zinc far above those required for inoculum growth. This permits use of media of improved nutritional balance, and the increased citric acid yields realized may result in part from this fact. The increased tolerance toward trace elements thus permits use of crude carbohydrate sources for citric acid production (38).

The effect of time of ethanol and methanol addition on citric acid production was shown in Table 4 and 5. The time interval was ranged from 0 to 75 h, after inoculation. Production of citric acid was increased with increase in time for addition of ethanol and methanol. Maximum citric acid production (44.62±2.12 g/l) was achieved with addition of 1.0 % ethanol into the fermentation media after 30 h of inoculation, while for methanol (1.5 %) addition maximum citric acid production (55.13±1.53 g/l) was observed after 45 h of inoculation. The amount of biomass formed and sugar consumed was

Table 2. Kinetic parameters and coefficients of citric acid fermentation by *A. niger* at different ethanol concentration

Parameters	Ethanol conc. (%)				
	0	0.5	1.0	1.5	2.0
Substrate uptake rate (Q_s)	0.639	0.652	0.664	0.673	0.622
Specific substrate uptake rate (q_s)	0.019	0.020	0.019	0.019	0.017
Product yield coefficient ($Y_{p/s}$)	0.221	0.234	0.318	0.276	0.265
Specific product yield coefficient ($Y_{p/x}$)	0.846	0.912	1.176	1.019	0.873
Growth yield coefficient ($Y_{x/s}$)	0.262	0.257	0.270	0.271	0.303
Productivity (Q_p)	0.141	0.152	0.211	0.186	0.165
Specific productivity (q_p)	0.004	0.004	0.006	0.005	0.004

Table representing kinetic parameters for citric acid production at different ethanol concentrations. Q_s = g substrate consumed/l/h, q_s = g substrate/g cells/l/h, $Y_{p/s}$ = (gram citric acid produced/gram sugar consumed), $Y_{p/x}$ = gram citric acid produced/ gram cells, $Y_{x/s}$ = (gram cell mass/gram sugar consumed), Q_p = g citric acid produced/l/h and q_p = (gram product/g cells/l/h). All values shown in this table differ significantly at $p \leq 0.05$.

Table 3. Kinetic parameters and coefficients of citric acid fermentation by *A. niger* at different methanol concentration

Parameters	Methanol conc. (%)				
	0	0.5	1.0	1.5	2.0
Substrate uptake rate (Q_s)	0.639	0.616	0.602	0.547	0.643
Specific substrate uptake rate (q_s)	0.019	0.018	0.016	0.014	0.016
Product yield coefficient ($Y_{p/s}$)	0.221	0.302	0.373	0.468	0.374
Specific product yield coefficient ($Y_{p/x}$)	0.846	1.045	1.150	1.277	1.180
Growth yield coefficient ($Y_{x/s}$)	0.262	0.289	0.324	0.366	0.317
Productivity (Q_p)	0.141	0.186	0.224	0.256	0.240
Specific productivity (q_p)	0.004	0.005	0.005	0.006	0.006

Table representing kinetic parameters for citric acid production at different methanol concentration. Q_s = g substrate consumed/l/h, q_s = g substrate/g cells/l/h, $Y_{p/s}$ = (gram citric acid produced/gram sugar consumed), $Y_{p/x}$ = gram citric acid produced/ gram cells, $Y_{x/s}$ = (gram cell mass/gram sugar consumed), Q_p = g citric acid produced/l/h and q_p = (gram product/g cells/l/h). All values shown in this table differs significantly at $p \leq 0.05$.

Table 4. Effect of Time of addition of ethanol to fermentation media on citric acid production

Time (Ethanol addition)	Citric acid (g/l)	Biomass (g/l)	Sugar consumed (g/l)	Yield (%)
0	40.85±0.48	32.87±0.53	123.5±3.45	33.07
15	42.53±2.45	34.52±1.03	127.6±2.62	33.33
30	44.62±2.12	35.23±1.12	130.4±4.51	34.21
45	39.15±1.97	34.25±0.73	132.5±3.57	29.54
60	36.27±2.63	35.63±1.35	130.4±1.82	27.81
75	34.62±0.78	33.87±1.03	119.6±2.62	28.94

Sugar added 150 g/l, temperature 30 ºC, initial pH 4.0±0.2, incubation period 8 days, shaking speed was 200 rpm, ethanol (1.0 %) was added after different time interval of incubation. The results are sum mean of three parallel replicates. ± indicates standard deviation among the replicates. All values shown in this table differs significantly at $p \leq 0.05$.

Table 5. Effect of time of addition of methanol to fermentation media on citric acid production

Time (Methanol addition)	Citric acid (g/l)	Biomass (g/l)	Sugar consumed (g/l)	Yield (%)
0	49.33±1.28	38.6±0.67	105.2±0.68	46.89
15	51.58±2.53	39.14±1.38	107.5±2.35	47.98
30	53.33±1.87	37.63±0.89	112.6±1.32	47.36
45	55.13±1.53	35.72±1.52	106.4±1.75	51.81
60	48.29±1.26	35.15±1.45	108.8±2.36	44.38
75	43.33±1.87	33.63±0.89	106.4±1.32	40.72

Sugar added 150 g/l, temperature 30 ºC, initial pH 4.0±0.2, incubation period 8 days, shaking speed was 200 rpm, methanol (1.5 %) was added after different time interval of incubation. The results are sum mean of three parallel replicates. ± indicates standard deviation among the replicates. All values shown in this table differs significantly at $p \leq 0.05$.

35.72±1.52 g/l and 106.4±1.75 g/l respectively under optimal time period for methanol addition into the fermentation media after inoculation. Further increase in time interval for both ethanol and methanol addition did no enhanced citric acid accumulation in fermentation media but a sudden decrease in terms of citric acid production was observed. Similar results were also reported by Haq et al. (33). According to them maximum citric acid production was achieved with addition methanol in to the fermentation media at different incubation periods but with further increase in incubation periods resulted decrease in citric acid accumulation.

On the basis of kinetic parameters as shown in Table 2 and 3, culture with ethanol and methanol as stimulatory agents was significantly improved for the values of $Y_{p/x}$ (0.912 g/g and 1.277 g/g) and $Y_{p/s}$ (0.234 g/g and 0.468 g/g) over the control medium (0.846 g/g and 0.221 g/g). All the parameters for citric acid production was much improved for culture grown on fermentation medium containing stimulatory agents as compared to the controlled medium as shown in Table 2 and 3. Significant specific citric acid productivity (0.256 g/l/h) was observed with the addition of 1.5 % methanol to the beet molasses medium. The stimulation of citric acid production by methanol in synthetic media is affected by cultural conditions, and especially by the mold strain used. The age and the amount of mycelia inoculum which is probably a reflection of its surface area may be critical. These factors must be investigated in applying the effect of the alcohol in any individual case (38).

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