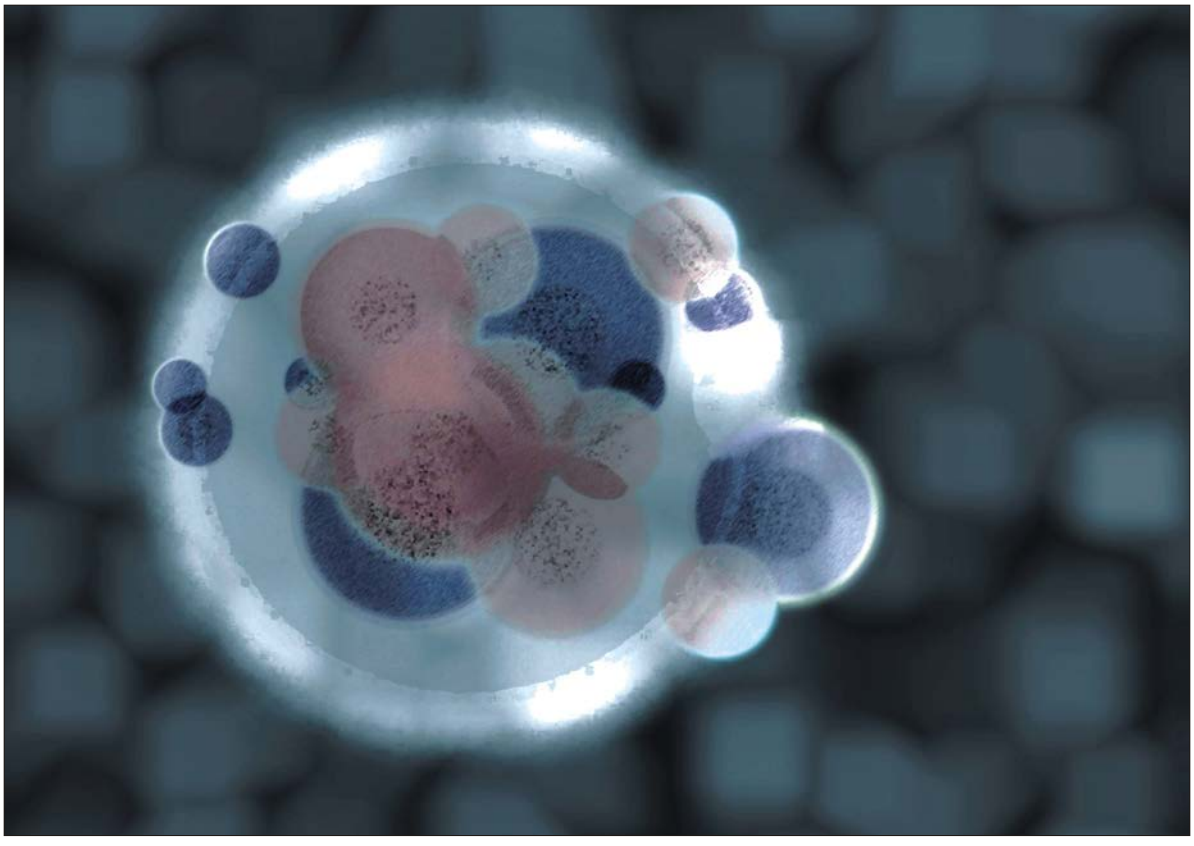

Journal of Biological Sciences

ISSN 1727-3048

science alert

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OnLine Journal of Biological Sciences 1 (4): 178-181, 2001

Biosynthesis of Citric Acid by Locally Isolated *Aspergillus niger* Using Sucrose Salt Media

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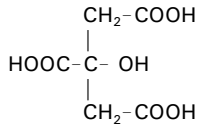
Abstract: Sixteen different cultures of *Aspergillus niger* were isolated from different soil samples. These isolates of *Aspergillus niger* were evaluated for citric acid fermentation in shake flask. Sucrose salt media was used and the volume of fermentation medium was kept at 25 ml. The cultural conditions such as pH (3.5), temperature (30°C), incubation period (8 days) and sugar concentration (15%), were optimised.

Key words: Citric acid, sucrose salt media, *Aspergillus niger* fermentation

Introduction

Citric acid is one of the most important bulk produced organic acid. Because of its commercial and academic importance, the biosynthesis of citric acid by moulds has been the subject of numerous investigations (Mattey, 1992). Environmental conditions markedly influence the growth pattern of filamentous fungi, which can range from a pellet to a dispersed filamentous form affecting in this way both the growth rate and the product formation (Pera and Callieri, 1999). Citric acid is the first stable intermediate compound of Kreb's cycle, having the following structure with three carboxylic groups;



(2-hydroxy propane 1, 2, 3-tricarboxylic acid)

The production of citric acid by *Aspergillus niger* is one of the most commercially utilised examples of fungal overflow metabolism (Kubicek and Rohr, 1977). The biosynthesis of citric acid by fermentation on commercial basis has been a worth praising achievement in the field of industrial microbiology. Because of its high solubility, palatability and low toxicity, it is one of the most commonly used acid in the food and pharmaceutical industries (Prescott and Dunns, 1987). A number of research reports have investigated on the submerged fermentation of citric acid by *Aspergillus niger* using sucrose salt media (Singh *et al*., 1998).

In this paper, we report the effect of different cultural conditions on the fungal growth pattern and citric acid production during submerged fermentation using sucrose salt medium by *Aspergillus niger* SIQ-14.

Materials and Methods

Different cultures of *Aspergillus niger* were isolated from soil samples of Lahore, by serial dilution method (Clark *et al*., 1958). These cultures were maintained on potato dextrose agar (PDA) slants, incubated at 30°C and stored at 4°C.

In the present study conidia from 3-5 days old cultures were used for inoculation. The conidial suspension was prepared in sterilized distilled water (10 ml) by gently scratching conidia with a sterile wire loop and then it was shaken vigorously for breaking the clumps of conidia.

The initial fermentation medium contained (%age w/v) sucrose, 15; KH_2PO_4, 0.1, $MgSO_4.7H_2O$, 0.025 and NH_4NO_3, 0.25. The optimum conditions for citric acid fermentation were investigated in 250 ml conical flask containing 25 ml of the medium having pH 3.5. One ml of conidial suspension was added to each flask. The flasks were rotated (200 rpm) in the incubator shaker at 30°C for 7 days.

Mycelial dry weight was determined by filtering the culture medium through weighed Whatman filter paper No. 44. The mycelium was thoroughly washed with tap water and dried at 110°C over night and mycelial dry weight was calculated.

The total acid was estimated by titrating 1.0 ml of fermented broth against 0.1N NaOH using phenolphthalein as an indicator.

$$\%\text{age total acid} = \frac{\text{Titre X Normality of alkali X Equivalent wt. of acid}}{\text{Volume of Sample X 1000}} \text{ X } 100$$

The anhydrous citric acid was determined colorimetrically according to the method of Marier and Boulet (1958).

The %age yield of citric acid was determined on the basis of sugar used, following the method of Suzuki *et al*. (1996).

$$\%\text{age of citric acid} = \frac{\text{Citric acid}}{\text{Sugar used}} \text{ X } 100$$

The sugar was estimated colorimetrically by DNS method as reported by Tasun *et al*. (1970).

Results

Table 1 shows the production of citric acid by locally isolated strains of *Aspergillus niger* , using sucrose salt media as substrate in shake flask. Sixteen different isolates of *Aspergillus niger* were isolated from soil and evaluated for citric acid production. These cultures were found to produce citric acid, ranging from 2.63 to 47.50 g/l. The culture of *Aspergillus niger* SIQ-14 was found to be the best producer of citric acid and it was selected for further studies.

The effect of volume of fermentation medium (10, 25, 50, 100 ml) on the citric acid production was carried out (Table 2). The maximum production of citric acid was obtained when the volume of fermentation medium was 25 ml. The consumption of sugar was 86 g/l and dry mycelial weight was 13.0 g/l. The %age of citric acid was 57.26% on the basis of sugar used and mycelia were intermediate round pellets in their morphology. The production of citric acid was decreased when the volume of the medium was further increased.

Table 3 shows the effect of different incubation periods on the production of citric acid by *Aspergillus niger* SIQ-14. The maximum production of citric acid was achieved after 7 days of inoculation. The sugar consumption was 87 g/l and dry mycelial weight was 11.5 g/l. The %age of citric acid on the basis of sugar used was 59.31% and the mycelial morphology was mixed pellets. Further increase in incubation period did not enhance citric acid production.

Table 1: Screening of Isolates of *Aspergillus niger* for the Production of Citric Acid Using Sucrose Salt Media

Isolates of *Aspergillus niger*	Citric Acid (g/l)
SIQ-1	9.77
SIQ-2	6.50
SIQ-3	11.16
SIQ-4	27.55
SIQ-5	2.63
SIQ-6	14.17
SIQ-7	19.91
SIQ-8	20.00
SIQ-9	12.75
SIQ-10	24.16
SIQ-11	31.22
SIQ-12	16.42
SIQ-13	29.10
SIQ-14	47.50
SIQ-15	25.00
SIQ-16	14.65

Temp. = 30°C Sugar = 150 g/l.
pH = 3.5

Table 4 shows the effect of different concentrations of sugar on the production of citric acid by *Aspergillus niger* SIQ-14 using sucrose salt media. The maximum amount of citric acid produced in the medium having 15% sugar. The consumption of sugar was 82 g/l and dry mycelial weight was 12.55 g/l. The %age of citric acid was 60.91% on the basis of sugar used and intermediate pellets of mycelium were formed.

Further increase in the concentration of sugar, resulted in gradual reduction in citric acid formation.

The effect of initial pH on citric acid production was carried out (Table 5). The pH of sucrose salt medium was ranged from 2.5-5.0. The maximum production of citric acid was achieved at pH 3.5. The sugar consumption was 85.0 g/l and dry mycelial weight was 12.55 g/l. The %age of citric acid on the basis of sugar used was 59.06% and the mycelia were intermediate round pellets. When the pH of fermentation medium was decreased or increased, the production of citric acid was decreased, gradually.

Table 2: Effect of Volume of Fermentation Medium on Citric Acid Production by *Aspergillus niger* SIQ-14

Volume of fermentation medium (ml)	Citric Acid (g/l)	Mycelial dry. weight (g/l)	Sugar (g/l)		% citric acid on the basis of sugar used	Mycelial morphology
			Used	Residual		
10	18.67	8.5	74	76	25.22	Gelatinous mass
25	49.25	13.0	86	64	57.26	Intermediate pellets
50	42.53	9.5	84	66	50.63	Large pellets
100	27.10	9.0	79.5	70.5	34.08	Elongated mycelia

pH = 3.5, Temp. = 30°C, Sugar = 150 g/l

Table 3: Effect of Incubation Period on the Production of Citric Acid by *Aspergillus niger* SIQ-14

Incubation period		Citric Acid (g/l)	Mycelial dry. weight (g/l)	Sugar (g/l)		% citric acid on the basis of sugar used	Mycelial morphology
Days	Hours			Used	Residual		
1	24	0.95	1.5	34	116	2.79	Viscous
2	48	3.1	2.5	45	105	6.89	Viscous
3	72	9.8	4.0	58	92	16.90	Very fine pellets
4	96	16.24	4.5	61	89	26.62	" " "
5	120	28.50	7.0	70	80	40.71	" " "
6	144	31.25	8.55	76	74	41.12	Small pellets
7	168	43.61	9.5	80	70	54.51	" " "
8	192	51.60	11.5	87	63	59.31	Mixed pellets
9	216	49.25	13.0	98	52	50.26	Large pellets
10	240	45.56	14.5	113	37	40.32	" " "

Temp. = 30°C, pH = 3.5, Sugar = 150 g/l

Table 4: Effect of Different Sugar Concentration on the Citric Acid Production by *Aspergillus niger* SIQ-14.

Sugar cone (%)	Citric Acid (g/l)	Mycelial dry. weight (g/l)	Sugar (g/l)		% citric acid on the basis of sugar used	Mycelial morphology
			Used	Residual		
9.0	12.0	8.0	47	43	25.53	Very fine pellets
12.0	26.52	10.05	80	40	33.15	Small pellets
15.0	49.95	12.55	82	68	60.91	Intermediate puts
18.0	43.20	14.60	131	49	32.98	" " "
21.0	37.55	16.0	165	45	22.76	Broken mycelia

Temp. = 30°C, pH = 3.5

Table 5: Effect of Different Ph of Fermentation Medium on the Production of Citric Acid by *Aspergillus niger* SIQ-14

pH	Citric Acid (g/l)	Mycelial dry. weight (g/l)	Sugar (g/l)		% citric acid on the basis of sugar used	Mycelial morphology
			Used	Residual		
2.5	24.50	13.5	79	71	31.01	Very fine pellets
3.0	34.00	13.0	82	68	41.46	" " "
3.5	50.20	12.55	85	65	59.06	Intermediate pellets
4.0	37.80	14.0	83	67	45.54	Mixed pellets
4.5	25.39	14.5	96	54	26.44	Large pellets
5.0	25.02	14.5	92	58	27.20	" " "

Temp. = 30°C, Sugar = 150 g/l

Table 6: Effect of Different Temperature on Citric Acid Production by *Aspergillus niger* SIQ-14

Temp. (°C)	Citric Acid (g/l)	Mycelial dry. weight (g/l)	Sugar (g/l)		% citric acid on the basis of sugar used	Mycelial morphology
			Used	Residual		
25	27.10	12.0	80	70	33.88	Small pellets
28	40.02	13.5	92	58	43.50	Small pellets
30	52.55	13.0	82	68	64.08	Mixed pellets
32	48.60	15.0	106	44	45.85	Large pellets
35	33.22	15.5	114	36	29.14	Gelatinous and fluffy mass

pH = 3.5, Sugar = 150 g/l.

The production of citric acid by *Aspergillus niger* SIQ-14 at different temperatures (25 - 35°C) was carried out (Table 6). The maximum amount of citric acid was achieved at 30°C. The sugar consumption was 82 g/l and dry mycelial weight was 13.0 g/l. The %age of citric acid was 64.08% on the basis of sugar used and mycelial morphology was mixed pellets. When the temperature of the medium was increased above 30°C, the production of citric acid was decreased.

Discussion

Citric acid, a tricarboxylic acid cycle intermediate, is one of the most important metabolic products produced commercially by fermentation with specific moulds but *Aspergillus niger* remains the organism of choice for citric acid fermentation (Pera and Callieri, 1997). The isolated mould cultures of *Aspergillus niger* produced citric acid ranging from 2.63-47.50 g/l. In the present study, we isolated 16 different cultures of *Aspergillus niger* showing insensitivity to catabolite repression and increased productivity of citric acid using sucrose salt medium. At present, we are examining citric acid production by the isolates of *Aspergillus niger* under various conditions.

The effect of different conditions such as volume of fermentation medium, incubation period, sugar concentration, pH and temperature on citric acid production by *Aspergillus niger* SIQ-14 was under-taken. Synthetic carbohydrate medium such as sucrose salt medium was employed as the basal substrate, for citric acid fermentation. Pera and Callieri (1999) also used synthetic media for biosynthesis of citric acid from *Aspergillus niger* strains. One of the main problems for achieving stable performance of citric acid fermentation with filamentous fungi involves limiting hyphal growth as well as avoiding diffusional restrictions (Brauer, 1990). The present study is important in showing that citric acid producing strains of *Aspergillus niger* with increased fermentation rates could be selected on the basis of different cultural conditions employed.

The maximum amount of citric acid was obtained when the volume of fermentation medium was 25 ml in 250 ml conical flasks. The production of citric acid was decreased when the volume of medium was further increased. It may be due to that when the volume of the medium was increased, the supply of air was reduced, which is necessary for production of citric acid. This finding is in agreement with the Dawson and Joloka (1983). Incubation period of 8 days was found to be the best for citric acid fermentation because further increase in inoculation time did not enhance citric acid production. It may be due to that above 8 days, the depletion of sugar contents and accumulation of other by-products take place, hence reduction in citric acid formation. This finding is in agreement with the observations of Singh *et al*. (1998). However Rajoka *et al*. (1998) got maximum production of citric acid 7 days after inoculation using molasses as basal medium in shake flask. The maximum amount of citric acid produced in the medium containing 150 g/l of sugar. Further increase in the concentration of sugar resulted in gradual reduction of citric acid formation. At low concentration of sugar, the mycelial growth was not well enough to produce citric acid, as a result the production of citric acid was reduced. Mattey and Allan (1990) described that with the increase of mycelial formation in the fermentation medium, there was reduction in the citric acid production. When the initial pH of sucrose salt medium was 3.5, the production of citric acid was highest. But any increase or decrease in pH value, greatly reduced citric acid biosynthesis. This is in agreement with the result of Pessoa *et al*. (1984) but is in contrast to those of Roukas and Harvey (1988) who reported that at low pH values citric acid is produced whereas at high pH values predominantly gluconic acid is formed. Citric acid production is directly related with the temperature of fermentation medium but upto a certain extent. The maximum production of citric acid was achieved at 30°C. When the temperature of the medium was increased above 30°C, the production of citric acid was decreased. Temperature above 30°C has inhibitory effect on citric acid formation and thus accumulation of other by-products such as oxalic acid take place (Srivasta and Kamal, 1979).

In summary, to obtain high productivity of citric acid, it is effective to obtain those isolates of *Aspergillus niger* which are sensitive to catabolite repression. Improvement of cultural condition and induction of the mutation in *Aspergillus niger* isolates may also increase the biosynthesis of citric acid in fermented broth.

Acknowledgements

The authors thank Pakistan Science Foundation (PSF), Islamabad, for financial and technical assistance.

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