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KINETICS OF IMPROVED CITRIC ACID PRODUCTION BY MUTANT STRAIN OF *ASPERGILLUS NIGER.*

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Abstract

The present investigation deals with the kinetics of improved citric acid production by *Aspergillus niger*. A laboratory scale stirred fermentor of 9-L working volume was used for cultivation. The mutant strain of *Aspergillus niger* NG-2 showed enhanced citrate activity (87.60 ± 0.3a g/l) over the parental strain GCB-75 (19.53 ± 0.2 g/l) and other mutant derivatives (49.85 ± 0.2a g/l citric acid in case of mutant UV-5 & 76.82 ± 0.6b g/l in case of mutant NG-7). The specific productivity of NG-2 (qp = 0.057 ± 0.05a g/g cells/h) was several folds higher than other strains. All other kinetic parameters including yield coefficients and volumetric rates also revealed the hyperproducibility of citric acid by using mutant NG-2 as the organism of choice.

Introduction

The increase in citric acid production by *Aspergillus niger* is subjected to various fermentation conditions and there have been attempts to improve citric acid productivity by mutation and polyploidy (Ajihade *et al.*, 1980). The idea of treating microorganisms with various mutagens and to search for improved mutants among surviving progeny has now been recognized as the best means to secure strains of improved citrate potency (Ewary, 1989: Haq *et al.*, 1998). Kresling and Stern (1935) reported that mutants of *Aspergillus niger* produce considerably more citric acid than the parent strain. But the feasibility of these observations was not realized till *Penicillium notatum*, which showed improved, yields of penicillin in comparison with parent strain. Since that time the process of induced mutation and strain selection has been used in improving the yield of various metabolic products (Shankaranand and Lonsane, 1993: Haq *et al.*, 2001).

Present study deals with the kinetics of improved production of citric acid by parent and mutant (UV-irradiated & chemically treated) strains of *Aspergillus niger*.

Materials and Methods

Organism and culture maintenance: The parent *Aspergillus niger* strain GCB-75 was used for citric acid fermentation. It was taken from the culture collection of Biotechnology Laboratories, Govt. College, Lahore. The culture was maintained on sterilized potato dextrose agar medium (Diced potato 200 g/l, Dextrose 20 g/l and Agar 15 g/l), pH 4.5. The slants of *Aspergillus niger* were stored at 5°C in the refrigerator.

Vegetative inoculum: Hundred ml of cane-molasses medium (Sugar 150 g/l, pH 6.0) containing glass beads, in 1-L cotton wool plugged conical flask was sterilized at 15-lbs/inch^2 pressure for 15 minutes. Small amount of conidia from the slant culture was aseptically transferred with the help of inoculating needle. The flask was incubated at 30°C in an incubator shaker at 200 rpm for 24 hours.

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Clarification of molasses: Cane molasses obtained from Chunian Sugar Mills (Pakistan) was clarified according to the method of Panda *et al.* (1984).

Fermentation technique: Stainless steel fermentor of 15 L capacity with working volume of 9 L was employed for citric acid fermentation. The fermentation medium consisting of (g/l); clarified cane molasses 300 (sugar 150 g/l), $K_4Fe(CN)_6$ 200 ppm and pH 6.0 was used for fermentation. All the culture media were sterilized at 121°C for 20 minutes. Vegetative inoculum was transferred to the production medium at a level of 5% (v/v). Incubation temperature was kept at 30±1°C throughout the fermentation period of 6 days. Agitation speed of the stirred was kept at 200 rpm and aeration rate was maintained at 1.0 l/l/min. Sterilized silicone oil was used to control the foaming problem during fermentation.

Ultraviolet and chemical mutation: The conidia of *Aspergillus niger* were UV-irradiated following the method of Haq *et al.* (2001). The cell suspension was treated with N-methyl N-nitro N-nitroso guanidine (NG) according to the procedure of Roy and Das (1977).

Assay methods: Mycelial dry weight' was determined according to Haq and Daud (1995). 'Sugar' was estimated colorimetrically by DNS method (1970). Photoelectric colorimeter model AE-11M (Erma, Japan) was used for measuring colour intensity. 'Anhydrous citric acid' was estimated using pyridine acetic anhydride method as reported by Marrier and Boulet (1958). The kinetics of the research work was studied after Pirt (1975).

Results and Discussion

Cultural conditions for citric acid production by fungi vary from strain to strain and also depend on the type of process (Karklins *et al.*, 1996; Ali *et al.*, 2001). Comparison of UV-irradiated and NG-treated mutants with parental strain of *Aspergillus niger*, for citric acid production is depicted in Table 1. The best UV-irradiated mutant strain UV-5 gave 2.82 folds higher production of citric acid (49.85 ± 0.2a g/l) as compared with parental GCB-75 (19.53 ± 0.2 g/l). Mould morphology was changed from fine round pellets to intermediate sized round pellets. However, N-methyl N-nitro N-nitroso guanidine (NG) treated mutants provoked better excretion of citric acid in the fermented broth i.e., NG-2 and NG-7 produced 87.60 ± 0.3a & 76.82 ± 0.6b g/l anhydrous citric acid, respectively. The best mutant strain of *Aspergillus niger* NG-2 gave 4.36 fold higher citrate activity. It might be due to the fact that NG is a strong mutagenic agent, which induces permanent changes in DNA structure, while UV-irradiated DNA recovered due to photo reactivation. Workers have also reported similar kind of observations (Ajihade *et al.*, 1980; Rajoka *et al.*, 1998). Among the kinetic parameters (yield coefficients, Yp/s, Yp/x & Yx/s in g/g) studied, the mutants showed enhanced citric acid productivity. The mutant NG-2 has highest values of all the yield coefficients (Table 2). Product yield coefficients (Yp/s & Yp/x) 1.002 ± 0.02a & 5.010 ± 0.04a (g/g), are several times improved as compared to Pirt (1975) and Rajoka *et al.* (1998).

The initial sugar concentration has been found to determine the amount of citric acid and also the amount of other organic acids produced by *Aspergillus niger*. In our finding, the mutant strain of *Aspergillus niger* NG-2 g

maximum yield of citric acid (88.66 ± 0.40a g/l) in the medium containing 150 g/l sugar. Gradual reduction in citric acid formation was observed when the sugar concentration of molasses medium was increased. It might be due to the over growth of mycelium, which resulted in increased viscosity of the medium and mass transfer limitation. Mattey and Allan (1990) described that with the increase of mycelial formation in the medium, there was reduction in the yield of citric acid. Kovats (1960) pointed out that a concentration higher than 15-18 %, however, leads to greater amount of residual sugars, making the process uneconomical; while a lower concentration of sugar leads to lower yields of citric acid as well as to the accumulation of oxalic acid.

The data of Table 4 shows the comparison of different kinetic parameters i. e., citric acid formation [Qp (g/l/h), Yp/s (g/g), Yp/x (g/g), qp (g/g cells/h)] and substrate consumption parameters [μ (h^{-1}), Yx/s (g cells/g), Qs (g/l/h), qs (g/g cells/h)] for the production of citric acid by *Aspergillus niger* and its mutant derivatives. All the parameters of citric acid production are much improved by mutant strain of *Aspergillus niger* NG-2 as compared with parental and other mutant derivatives. The specific citrate productivity of mutant NG-2 was 0.057 ± 0.05a, which is several folds higher than the other strains. On the other hand, specific substrate uptake rate i. e., qs (g/g cells/h) = 0.035 ± 0.03bc is also very significant.

The optimum time of incubation for maximal citric acid production varies both with the organism and fermentation conditions. The rate of citric acid synthesis was studied and the maximum yield of citric acid (93.15 ± 0.2a g/l) was achieved, 6 days after inoculation with mutant NG-2 (Figures I & II). The mutant strain of *Aspergillus niger* NG-7 also provoked better excretion of citric acid (78.12 g/l) but consumption of sugar was higher in this case i. e., Yx/s (g/g) = 1.86 ± 0.03b (Figure III). In batch-wise fermentation, citric acid production starts after a lag phase of one day and reached maximum at the onset of stationary phase. Further increase in incubation period did not enhance citric acid production. It might be due to the decrease in amount of available nitrogen in fermentation medium, age of fungi and depletion of sugar contents. Vergano *et al.* (1996) reported the maximum yield of citric acid, 7 days after the inoculation. Clark (1962) obtained about 80 % conversion of available sugar in 8 days of fermentation. So our finding is more significant as compared to these workers.

Conclusion

The best NG-treated mutant strain of *Aspergillus niger* NG-2 provoked better production of citric acid in laboratory scale stirred fermentor. The product is low volume, high cost product. All the product yield coefficients (Yp/s, Yp/x & Yx/s in g/g), volumetric rates (Qp, Qs & Qx in g/g cells/h) and specific rate constants are higher than many of the other workers. The specific productivity of NG-2 i. e., qp = 0.057 ± 0.05a is highly significant. By more quantitative analysis of product formation and optimisation of fermentation conditions such as the effect of nitrogen and magnesium sources on fungal growth, this strain can be well exploited for industrial-scale production of citric acid.

Table 1: Comparison of UV-irradiated and NG-treated mutants with parental strain of *Aspergillus niger* for citric acid production.

Aspergillus niger strains	Mycelial dry weight (g/l)	Glucose consumed (g/l)	Anhydrous citric acid (g/l)	Mycelial morphology
Parental strain				
GCB-75	18.50	112.50	19.53 ± 0.2	Fine round pellets
UV-irradiated mutant strains				
UV-1	15.65	98.26	28.12 ± 0.1c	Viscous
UV-2	15.40	116.15	12.80 ± 0.4d	Viscous
UV-3	19.15	89.24	5.76 ± 0.2e	Large broken mycelia
UV-4	25.75	134.10	29.62 ± 0.3bc	Small pellets
UV-5	16.90	92.86	49.85 ± 0.2a	Intermediate size round pellets
UV-6	29.10	111.05	37.13 ± 0.4b	Mixed pellets
NG-treated mutant strains				
NG-1	18.25	107.75	62.18 ± 0.5c	Intermediate pellets
NG-2	17.50	87.62	87.60 ± 0.3a	Intermediate pellets with shiny surfaces
NG-3	22.55	139.16	42.42 ± 0.5e	Small laxly pellets
NG-4	26.10	142.56	46.55 ± 0.4de	Small laxly pellets
NG-5	16.15	102.05	51.26 ± 0.4d	Elongated mycelia
NG-6	16.05	97.18	13.66 ± 0.2f	Gummy mass
NG-7	17.95	89.15	76.82 ± 0.6b	Intermediate pellets having rough surfaces
NG-8	24.35	121.42	59.80 ± 0.5cd	Intermediate pellets in viscously substance

All the fermentations were carried out at 30°C following growth on 150 g/l initial sugar concentration. The initial pH of the molasses medium was kept constant at 6.0 throughout the fermentation period of 6-days.

The results are sum mean of three parallel replicates. ± indicates standard deviation among the replicates. Values for citric acid production differ by letters at $p \leq 0.2$.

Table 2: Production of citric acid by mutants of *Aspergillus niger* obtained through UV-irradiation and NG-treatment.

Strains tested	Citric acid (g/l)	Kinetic parameters (g/g)		
		Yp/s	Yp/x	Yx/s
Parental strain				
GCB-75	19.86 ± 0.3d	0.174 ± 0.02d	1.056 ± 0.07d	0.164 ± 0.01d
UV-derived best mutant strain				
UV-5	51.62 ± 0.3c	0.537 ± 0.03c	2.950 ± 0.04c	0.182 ± 0.02c
NG-derived 2-best mutant strains				
NG-2	87.44 ± 0.2a	1.002 ± 0.02a	5.010 ± 0.04a	0.200 ± 0.02a
NG-7	79.18 ± 0.5b	0.862 ± 0.02b	4.280 ± 0.06b	0.201 ± 0.01a

Kinetic parameters:

Yp/s (g/g) = citric acid produced (g/l) / substrate consumed (g/l), Yp/x (g/g) = citric acid produced (g/l) / cell mass formed (g/l), Yx/s (g/g) = cell mass formed (g/l) / substrate consumed (g/l).

The values differ significantly by letters at $p \leq 0.05$. ± indicates standard deviation among the replicates.

Table 3: Effect of different concentration of molasses sugar on citric acid production by mutant strain of *Aspergillus niger* NG-2.

Molasses sugar (g/l)	Mycelial dry weight (g/l)	Sugar consumed (g/l)	Anhydrous citric acid (g/l)	Qx (g cells/l/h)	% citric acid yield	Mycelial morphology
60	9.50	46.55	13.15 ± 0.4ef	0.065 ± 0.02f	28.25	Fine pellets
90	13.25	67.90	29.84 ± 0.3e	0.092 ± 0.02de	43.95	Laxly small pellets
120	14.10	90.15	55.12 ± 0.3b	0.098 ± 0.05d	61.14	Mixed pellets
150	18.50	98.20	88.66 ± 0.4a	0.128 ± 0.04c	90.28	Mixed mycelia
180	26.60	126.50	48.12 ± 0.2c	0.185 ± 0.02a	38.04	Gelatinous
210	21.75	145.05	43.71 ± 0.7d	0.151 ± 0.03b	30.13	mass Gummy mass

Initial pH 6.0, incubation temperature 30°C.
Qx = volumetric rate of cell mass formation.
± indicates standard deviation among the replicates. Values for citric acid production differ by letters at $p \leq 0.02$.

Table 4: Kinetic parameters for production of citric acid from 150 g/l molasses carbohydrate following growth of *Aspergillus niger* and its mutant derivatives.

Kinetic parameter	Parental strain GCB-75	UV-irradiated mutant strain UV-5	NG-treated mutant strains	
			NG-2	NG-7
Citric acid formation parameters				
Qp (g/l/h)	0.136 ± 0.02d	0.346 ± 0.02c	0.608 ± 0.04a	0.533 ± 0.03ab
Yp/s (g/g)	0.174 ± 0.02d	0.537 ± 0.03c	1.000 ± 0.03a	0.862 ± 0.01b
Yp/x (g/g)	1.056 ± 0.01d	2.950 ± 0.02c	5.010 ± 0.04a	4.280 ± 0.02b
qp (g/g cells/h)	0.007 ± 0.04d	0.020 ± 0.02c	0.057 ± 0.05a	0.030 ± 0.04bc
Substrate consumption parameters				
μ (h^{-1})	0.128 ± 0.01a	0.117 ± 0.02c	0.122 ± 0.02bc	0.125 ± 0.02ab
Yx/s (g cells/g)	0.164 ± 0.01c	0.182 ± 0.03b	0.200 ± 0.03a	0.201 ± 0.03c
Qs (g/l/h)	0.781 ± 0.03a	0.645 ± 0.04b	0.608 ± 0.04c	0.619 ± 0.04c
qs (g/g cells/h)	0.042 ± 0.01a	0.038 ± 0.02b	0.035 ± 0.03bc	0.034 ± 0.02c

Each value is an average of three replicates. ± Indicates standard deviation among replicates. The numbers differ significantly by $p \geq 0.05$. μ (h^{-1}) = specific growth rate, Yx/s = g cells/g substrate utilized, Qs = g substrate consumed/l/h, qs = g substrate consumed/g cells/h, Qp = g citric acid produced/litre/h, Yp/s = g citric acid produced/g substrate consumed, Yp/x = g citric acid produced/g cells formed, qp = g citric acid produced/g cells/h.

Figure 1. Citric acid production by mutant strain of *Aspergillus niger* NG-2 in stirred fermentor. The fermentation was carried out at 30°C. Initial sugar concentration and pH were 150 g/l and 6.0, respectively.

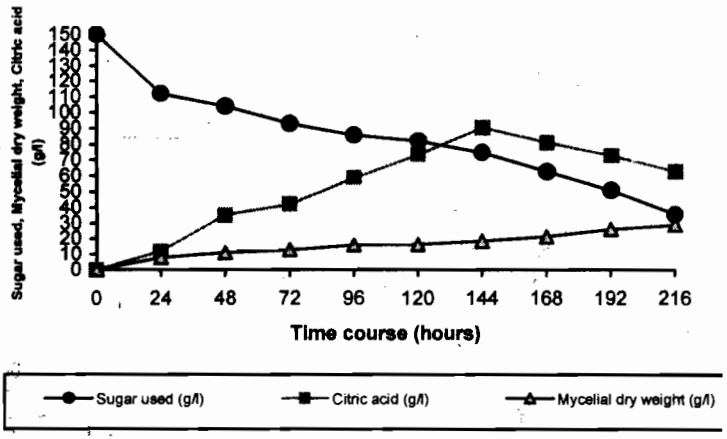


Figure 2. Citric acid production by mutant strain of *Aspergillus niger* NG-7 in stirred fermentor. The fermentation was carried out at 30°C. Initial sugar concentration and pH were 150 g/l and 6.0, respectively.

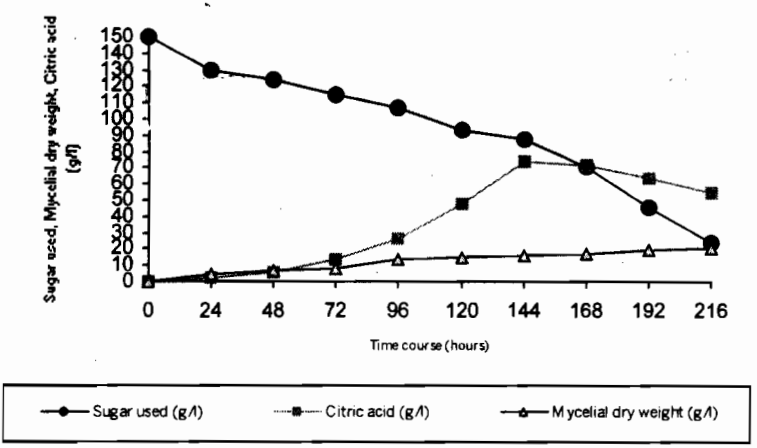
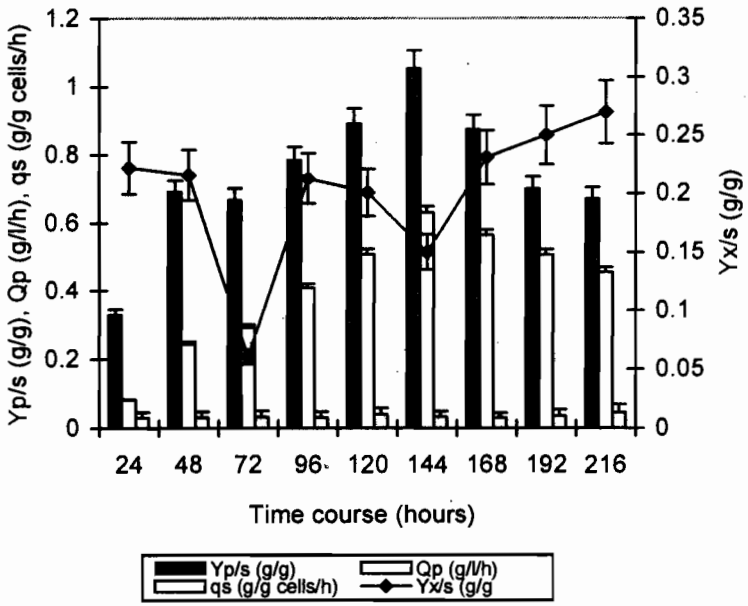


Figure 3. Comparison of kinetic parameters (Yp/s, Yx/s in g/g, Qp in g/l/h & qs in g/g cells/h) for citric acid production by mutant NG-2 in stirred fermentor.



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