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NITROGEN LIMITATION FOR ENHANCED CITRIC ACID PRODUCTIVITY BY A 2-DEOXY D-GLUCOSE RESISTANT CULTURE OF *ASPERGILLUS NIGER* NG^{D}-280

SIKANDER ALI, IKRAM-UL-HAQ, M.A. QADEER AND [1]JAVED IQBAL

Biotechnology Research Laboratory,
Department of Botany, Government College, Lahore, Pakistan.

Abstract

The present investigation is concerned with the nitrogen limitation for enhanced citrate productivity by a 2-deoxy D-glucose resistant culture of *Aspergillus niger* NGd-280 in 15-L stirred tank bioreactor. Nutrients especially nitrogen source have a marked influence on citrate productivity because it is an essential constituent of basal cell proteins. Ammonium nitrate at various concentrations was used as a nitrogen source. The specific growth rate was inhibited and the biosynthesis of citric acid was delayed at higher concentration of ammonium nitrate. The specific citric acid production rate was highest when intracellular ammonium ion concentration was between 2.0-3.0 mmol/g cells. However, citrate production was stopped when intracellular ammonium ion concentration decreased below 1.0 mmol/g cell.

Introduction

Among the microorganisms that produce citric acid, *Aspergillus niger* is one of the most well known producers. Citric acid was first isolated from lemon juice and crystallized as calcium citrate, but now fermentation processes mainly produce it (Pazouki *et al.*, 2000). Considerable efforts have been made towards the improvement of fermentation processes as well as the elucidation of microbial fundamentals. Research has also been made on the nutrients and oxygen transfer effects and other environmental factors related with citric acid production (Anon., 1975, Ajihade *et al.*, 1980). When oxygen is enough citric acid is produced, while it is known to be consumed by the microorganism especially *Aspergillus niger* when oxygen is depleted. Results have reported novel bioreactor design such as an immobilized cell or fluidized bed bioreactor (Arzumanov *et al.*, 2000). Carbon and nitrogen sources are known to be important as inorganic sources such as ferrous and manganese. Citric acid production is affected by the regulatory role of the ammonium ion. The present study deals with the nitrogen limitation for enhanced citric acid production by a 2-deoxy D-glucose resistant strain of *Aspergillus niger* NG^{d}-280 using a laboratory scale stirred tank bioreactor. The effect of ammonium nitrate as nitrogen source on citric acid fermentation, at low level has also been investigated on kinetic basis.

Materials and Methods

Organism and culture maintenance: *Aspergillus niger* strain NG^{d}-280 obtained from the culture collection of *Biotechnology Labs., Govt. College, Lahore* was used for citric acid fermentation. The culture was maintained on solidified potato dextrose agar medium (Merck, Germany) pH 4.5, previously stabilized by 0.026 % 2-deoxy D-glucose addition and improved by 0.015 % N-methyl N-nitro N-nitroso guanidine treatment. The slants of *A. niger* were stored at 5°C in the refrigerator.

[1]Department of Botany, Quaid-e-Azam Campus, University of the Punjab, Lahore, Pakistan.

Table 1. Time course of citric acid fermentation (initial ammonium nitrate concentration was 0.30 gl^{-1}) by *Aspergillus niger* NG^{d}-280 in stirred fermentor.

Incubation period (hours)	Dry cell mass (gl^{-1})	Citric acid (gl^{-1})	Sugar consumption (gl^{-1})	Residual NH_4NO_3 (gl^{-1})
0	0	0	150	0.30
24	4.10	2.02	136	0.26
48	5.30	8.56	124	0.22
72	6.85	14.66	108	0.17
96	9.30	41.65	94	0.14
120	11.80	69.97	70	0.13
144	14.95	112.54	62	0.12
168	16.55	77.12	51	0.01
192	17.70	56.52	34	0.01

Sugar added 150 gl^{-1}, aeration rate 1.0$ll^{-1}min^{-1}$ and initial pH 6.0. The results are sum mean of three parallel replicates.

Table 2. Effect of ammonium nitrate concentration on the specific growth rate μ (h^{-1}) and specific product formation constant Qp ($gl^{-1}h^{-1}$) by *Aspergillus niger* NG^{d}-280.

NH_4NO_3 concentration (gl^{-1})	μ (h^{-1})	Qp ($gl^{-1}h^{-1}$)	Probability < p >
0	0.343±0.02	0.124±0.01	-
0.1	1.421±0.02	0.242±0.01	-
0.2	1.718±0.03	0.401±0.01	-
0.3	2.452±0.04	0.611±0.01	HS
0.4	1.420±0.04	0.220±0.02	-
LSD	0.284	0.206	

The ± indicates standard error of mean among the three parallel replicates. The values differ significantly at $p < 0.05$. LSD is for least significant difference while HS denotes that the values are highly significant.

Vegetative inoculum and molasses clarification: Hundred ml of molasses medium (Sugar 15%, pH 6.0) containing silica gel chips (1.2 mm, diam.,), in 1 litre cotton wool plugged conical flask, was sterilized at 15 $lbs/inch^2$ pressure (121°C) 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 (Gallenkamp, UK) at 200 rpm for 24 hours. The vegetative inoculum was transferred to the production medium at a level of 5% (v/v) based on total working volume of the fermentation medium. Cane molasses obtained from Chunian Sugar Mills (Pakistan) was clarified according to the method of Panda *et al.*, (1984).

Fermentation technique: A stainless steel stirred fermentor (GLSC-101) of 15-L capacity with working volume of 9-L was employed for citric acid fermentation. The fermentation medium consisting of (gl^{-1}); clarified cane molasses 300 ml (sugar 15%), $K_4Fe(CN)_6$ 200 ppm at pH 6.0 was used for fermentation. The NH4NO3 was added during medium preparation. All the culture media were sterilized at 121°C for 15 minutes. The incubation temperature was kept at 30±1°C throughout the fermentation period of 144 hours. Agitation speed was 200 rpm while aeration rate was maintained at 1.0 $ll^{-1}min^{-1}$. Sterilized silicone oil (AE-11) was used to control foaming.

Table 3. Intracellular ammonium ion concentration during citric acid fermentation by *Aspergillus niger* NG^{d}-280.

Incubation period (hours)	Citric acid (gl^{-1})	Residual NH_4NO_3 (gl^{-1})	Intracellular ammonium ions (mmol/g cells)
0	0	0.30	0.40
24	6.23	0.25	0.39
48	9.46	0.24	0.37
72	19.75	0.20	0.37
96	50.15	0.16	0.36
120	72.52	0.14	0.31
144	113.10	0.12	0.27
168	80.16	0.01	0.24
192	62.06	0.01	0.19

Temperature 30°C, Sugar added 150gl^{-1}, aeration rate 1.0$ll^{-1}min^{-1}$, initial pH 6.0.
The results are sum mean of three parallel replicates.

Table 4. Comparison of kinetic parameters for citric acid fermentation at different ammonium nitrate concentration.

Parameters	Initial ammonium nitrate concentration (gl^{-1})				LSD
	0.10	0.20	0.30	0.40	
Maximum cell mass (gl^{-1})	8.80±1.2	12.20±1.4	19.00±1.4	16.00±1.8	-
Maximum citric acid concentration (gl^{-1})	38.12±4.6	80.54±3.2	114.05±3.6	63.66±6.8	-
Time for ammonium nitrate consumption (h)	72	96	144	192	-
Specific uptake rate of ammonium nitrate (mg/g cell/h)	8.34±0.4	13.51±0.4	27.68±0.6	18.29±0.5	3.28
Volumetric productivity ($gl^{-1}h^{-1}$)	0.80±0.1	1.70±0.1	2.20±0.2	1.20±0.4	0.172
Probability	**HS**	**HS**	**HS**	**HS**	

Aeration rate 1.0$ll^{-1}min^{-1}$, initial pH 6.0.
The ± indicates standard error of mean among the three parallel replicates. Values differ significantly at $p < 0.05$. LSD is for least significant difference while HS denotes that the values are highly significant.

Analysis: Mycelial dry weight was determined according to Haq & Daud (1995). Sugar was estimated colorimetrically by DNS method (Tasun *et al.*, 1970). A scanning UV/VIS spectrophotometer (Cecil-700, UK) was used for measuring % colour intensity. Anhydrous citric acid was estimated using pyridine acetic-anhydride method (Marrier & Boulet, 1958).

Kinetical and statistical studies: The kinetics of the research work was studied after Pirt (1975). Statistical analyses of the data were determined following the procedures of Snedecor & Cochren (1980). Standard deviation among the replicates was presented in the form of probability (< p >) values.

Results and Discussion

Being the basic part of cell protein, all microorganisms require nitrogen (Haq & Daud 1995). Ammonium nitrate was used as a nitrogen source in the present study. Initial pH is very important in citric acid fermentation and can be maintained at a low level by using ammonium nitrate as a nitrogen source. Nitrate is known to be converted to nitrite and finally to ammonium ion inside the cell (Roukas & Harvey 1988). Time course of citric acid fermentation when the initial ammonium nitrate concentration was 0.3 gl^{-1} is shown in Table 1. Sucrose consumption was continued with the increase of cell mass during citric acid biosynthesis. A total 112.54 gl^{-1} anhydrous citric acid was produced after 144 hours of incubation. A series of batch cultures were conducted with different initial ammonium nitrate concentration (0.1-0.4 gl^{-1}) (Table 2). Ammonium nitrate showed a substrate inhibition on the cell growth. Optimum ammonium nitrate concentration for the cell growth was 0.3 gl^{-1} under the experimental conditions. The inhibition was known to be caused by intracellular nitrite converted from nitrate (Ali *et al.*, 2001).

The relation between the intracellular ammonium ions and citric acid biosynthesis was investigated in the batch culture experiment 0.1 gl^{-1}. When the concentration of intracellular ammonium ion was between 0.2-0.3 mmol/g cell, the production rate of citric acid was highest (Table 3). When the concentration of intracellular ammonium ion decreased below 0.1 mmol/g cell, the citric acid production almost stopped. From the results it can be postulated that nitrogen source is required for maintaining the cell activity and also for the prevention of conidial formation. The results are substantiated with the findings of Kristiansen & Sinclair (1979). The experimental results of 4-batch run at different initial ammonium nitrate concentrations have been shown in Table 4. Optimum cell mass growth and maximum citric acid concentrations were obtained when the initial ammonium ion concentration was 30 gl^{-1}. At this concentration, the overall specific uptake rate of ammonium nitrate was high and it took longer for the ammonium nitrate to be consumed. After ammonium nitrate had almost been consumed, an increase of cell mass was still observed and biosynthesis of citric acid was almost stopped. The presence of intracellular ammonium ions was thus considered in the present study. Some of the ammonium nitrate that was taken up (intracellular), might be converted to amino acid for the growth of the cell after the depletion of ammonium ions which acted as regulator for citrate biosynthesis (Ajihade *et al.*, 1980).

Conclusion: Ammonium nitrate at a level of 0.30 gl^{-1} is found to be optimum for maximum production of citric acid (113.10 gl^{-1}) in the present study. The intracellular ammonium ions (0.27 mmol/g cells) is directly related with mycelial morphology being the basal part of cell proteins and subsequently citric acid production. The enhancement is substantial and is of industrial level. The volumetric productivity i.e., 2.20±0.2 $gl^{-1}h^{-1}$ is highly significant. However, further work on the effect of NH_4 ions on rheological properties and partial purification of citric acid from the culture broth is in progress.

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