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CONTROL OF *ASPERGILLUS NIGER* MORPHOLOGY TO ENHANCE CITRIC ACID PRODUCTION UNDER LIQUID CULTURE

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Abstract

The present investigation deals with the control of *Aspergillus niger* morphology to enhance citric acid production under liquid culture. For this purpose, the parental *Aspergillus niger* GCB-16 and its mutant derivative NG-101 were mutually compared, using 150 g/l carbohydrates of cane-molasses as the basal fermentation medium. The mutant showed a 1.26 fold increase in citric acid production over the control as compared to 2.82 in times the wild-type culture. Addition of 2.0 x 10^{-5}M $CuSO_4$ to the fermentation medium reduced the Fe^{+2} ion concentration by counteracting its deleterious effect on fungal growth. The copper ion also induced a loose-pelleted form of growth (0.5 mm, dia.), reduced the biomass concentration (12.5 g/l) and increased the volumetric productivity of citric acid monohydrate (93.6±5 g/l). On the basis of comparison of kinetic parameters viz., the volumetric substrate uptake rate (Qs) and specific uptake rate (qs), the volumetric productivity, theoretical yield and specific product formation rate, it was observed that the mutant was faster growing organism (Yx/s = 0.118±0.02 g/g) and had the ability to overproduce citric acid (Yp/s = 0.340±0.02 g/g).

Introduction

In liquid cultures, it is the mycelial morphology moulds which determines the citrate synthase ability of the microorganism especially *Aspergillus niger*. Cane-molasses, a bye-product of sugar industry has about 45-60% carbohydrate content, thus making it a useful medium for citric acid fermentation. Citric acid produced by *Aspergillus niger* is extremely sensitive to heavy metals present in molasses such as iron, zinc, copper and manganese in submerged fermentation (Majolli & Aguirre, 1999; Pera & Callieri, 1997). The concentration of these heavy metals, therefore, should be decreased well below that required for optimal mould growth (Maria & Wladyslaw, 1989). The optimum concentration of Fe^{++} required for maximal citric acid production has been found to vary with the strain of the fungus. It is reported that citric acid production by *Aspergillus niger* under submerged fermentation conditions using molasses as the substrate is severely affected by the presence of iron at a concentration as low as 0.2 ppm (Fedoseev, 1970). Present investigation deals with the increase in citric acid production by reducing Fe^{2+} concentration in blackstrap molasses under liquid culture.

Materials and Methods

Organisms: The parent *Aspergillus niger* strain GCB-16 and its mutant derivative strain NG-101 were used for citric acid fermentation. These cultures were obtained from the culture collection of Biotechnology Research Laboratories, Department of Botany, Govt. College, Lahore, Pakistan and maintained on potato dextrose agar medium, pH 4.5 (Fluka, Switzerland). The cultures were stored at 4°C in a refrigerator.

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Cane-molasses pre-treatment: The cane-molasses (*Medina Sugar Mills, Hafizabad*) was clarified and maintained at 15% sugar level. Initial pH was adjusted at 6.0 by 0.1N HCl / 0.1N $Ca(OH)_2$.

Fermentation conditions: Vegetative inoculum used in the present study was developed following the method of Khan *et al.*, (1970). A stainless steel stirred fermentor (Model: GLSC-AF-199-10) of 15-litre capacity with working volume of 9-litre was employed for fermentation. The fermentation medium consisting of clarified cane-molasses: sugar 15%, NH_4NO_3 0.025%, ash contents 0.45%, trace metals like iron, manganese, zinc 0.065% and $K_4Fe(CN)_6$ 200 ppm at pH 6.0 was sterilized at 15 lbs/inch^2 pressure (121°C) for 15 minutes. The copper salts ($CuSO_4$, $CuCl_2$, $CuNO_3$, $CuCO_3$) were added at a level of 2.0×10^{-5} M at the time of inoculation. The inoculum was used at a level of 5% (v/v). Incubation temperature was kept at 30°C for 144 hours. Agitation speed and aeration rate were kept at 200 rev./min and 1.0 l/l/min, respectively. Sterilized silicone oil (6.5%) was used to control the foaming. Samples were taken after every 24 hours. All experiments were run parallel in triplicates.

Assay methods: Dry cell mass was determined by filtering the fermented broth through weighed Whatman filter paper No. 44. The filtrate was used for further analysis while mycelium was thoroughly washed with tap water and dried at 110°C overnight. Citric acid was determined colorimetrically using pyridine-acetic anhydride method (Marrier & Boulet, 1958) and residual sugar was estimated by a DNS method (Tasun *et al.*, 1970).

Data kinetics and statistical tests: The kinetic parameters were studied after Pirt (1975). Data was statistically analysed for Duncan's multiple range tests and one-way ANOVA (SW/spss-*10, version 4.0*) following the methods of Kubicek & Rohr (1985).

Results and Discussion

In liquid cultures, the mycelial morphology and pellet size was directly related with citric acid production. For this purpose Cu^{++} can help by reducing Fe^{++} ion concentration in blackstrap molasses medium. The effect of different copper sources and their concentrations on the production of citric acid by parental strain of *Aspergillus niger* GCB-16 and its mutant derivative NG-101 showed that copper sulphate @ 2.0×10^{-5}M gave maximum production of (92.0±5 g/l) of citric acid monohydrate (Table 1). This might be due to the fact that $CuSO_4$ gives free Cu^{2+} ions in the production medium which are not only beneficial for fungal growth but may also counter-act the deleterious effects of Fe^{2+} on mycelial development. The presence of Cu^{2+} ions in the medium also induced the pellet like morphology of fungal mycelium. At low concentration of Cu^{2+} ions (1.0×10^{-5}M), the production of citric acid was also low (84.6±3 g/l). The presence of $CuSO_4.5H_2O$ higher than 2.0×10^{-5}M did not show any effect on the structure and morphology of the pellets, but significantly decreased (81.5±3 & 70.6±2 g/l) citric acid production. Kubicek & Rohr (1985) and Khan *et al.*, (1970) believed that Cu^{2+} may stimulate citric acid production by inhibiting aconitase whereas others reject this possibility, finding no alterations in the citrate-isocitrate ratio on adding Cu^{2+} under the conditions of citric acid production. However, favourable changes in citric acid yield were observed.

Table 1. The influence of different copper sources and their concentration on the production of citric acid by *Aspergillus niger* GCB-16 (parental strain) and NG-101 (mutant strain).

Copper sources	Concentration of copper sources (a x 10^{-5}M)	Citric acid monohydrate (g/l)		Sugar consumption (g/l)		Dry cell mass (g/l)		Mycelial Morphology	
		GCB-16	NG-101	GCB-16	NG-101	GCB-16	NG-101	GCB-16	NG-101
Control		32.4±3	75.4±4	95.2	86.5	9.0	10.2	Small pellets	Intermediate pellets
Copper sulphate	1.0×10^{-5}M	33.8±1	84.6±3	91.5	88.2	9.5	13.0	Mixed pellets	Small pellets
	2.0×10^{-5}M	39.4±2	92.0±5	89.2	84.6	10.5	12.5	Mixed pellets	Mixed pellets
	3.0×10^{-5}M	38.2±2	81.5±3	87.5	82.0	12.4	16.2	Laxy pellets	Mixed pellets
	4.0×10^{-5}M	31.0±5	70.6±2	86.2	81.2	9.2	12.0	Small pellets	Small pellets
Copper chloride	1.0×10^{-5}M	26.1±3	51.6±2	91.5	90.2	7.0	8.6	Large pellets	Last mycelia
	2.0×10^{-5}M	29.0±4	59.0±1	105.5	91.6	9.8	10.6	Broken pellets	Small pellets
	3.0×10^{-5}M	21.1±3	54.2±2	108.2	87.6	9.5	12.0	Gummy mass	Small pellets
	4.0×10^{-5}M	18.2±3	52.5±3	101.4	86.0	7.6	14.0	Gummy mass	Small pellets
Copper nitrate	1.0×10^{-5}M	25.5±4	61.0±4	112.5	101.6	12.1	11.8	Viscous	Gummy mass
	2.0×10^{-5}M	27.4±4	65.6±4	116.8	109.8	11.6	10.8	Viscous	Gummy mass
	3.0×10^{-5}M	18.8±1	64.2±4	121.6	110.8	9.2	9.6	Viscous	Gelatinous
	4.0×10^{-5}M	12.4±2	62.8±3	102.8	106.5	9.9	9.0	Viscous	Gelatinous
Copper carbonate	1.0×10^{-5}M	29.8±2	41.2±5	100.2	100.0	15.0	13.2	Gummy mass	Viscous
	2.0×10^{-5}M	34.2±3	56.0±4	96.2	101.8	9.4	10.6	Fluffy mycelia	Viscous
	3.0×10^{-5}M	31.2±2	50.4±2	81.6	92.6	9.0	9.8	Fluffy mycelia	Viscous
	4.0×10^{-5}M	29.0±1	45.2±3	80.2	83.6	8.0	9.3	Gelatinous	Fine pellets

Cultural conditions: temperature 30°C, aeration rate 1.0 litre/litre/min, initial sugar concentration 150 g/l, initial pH 6.0. Each value is an average of three replicates. The symbol ± shows the standard deviation among the replicates. The numbers differ significantly by $p<0.05$.

Table 2. Effect of time of addition of copper sulphate on citric acid production and mycelial morphology by wild-strain of *Aspergillus niger* and its mutant derivative.

Time of addition of $CuSO_4.5H_2O$ (hours)	Citric acid monohydrate (g/l)		Mycelial morphology	
	GCB-16	NG-101	GCB-16	NG-101
Control	35.5±2	78.2±4	Mixed pellets	Intermediate round pellets
0	38.6±2	84.0±3	Mixed pellets	Intermediate round pellets
6	42.8±3	93.6±5	Mixed pellets	Mixed laxy pellets
12	31.5±4	88.4±3	Large pellets	Mixed pellets
18	30.4±2	81.0±2	Large pellets & elongated hyphae	Large pellets
24	30.2±3	76.8±4	Gummy mass	Large broken pellets

Cultural conditions: copper sulphate concentration 2.0×10^{-5}M, sugar added 150 g/l, agitation intensity 160 rev./min. The numbers differ significantly at $p<0.05$. The sign ± indicates standard deviation among the three parallel replicates.

Table 3. Kinetic parameters for production of citric acid from sugars in molasses following growth of *Aspergillus niger* and its mutant derivative.

Kinetic parameters	Control without copper sulphate		with copper sulphate @ 2.0×10^{-5}M	
	Parental strain GCB-16	Mutant NG-101	Parental strain GCB-16	Mutant NG-101
Substrate consumption parameters				
μ (h^{-1})				
Yx/s (g cells/g)	0.193±0.02	0.449±0.01	0.234±0.02	0.548±0.03
Qs (g/l/h)	0.094±0.03	0.118±0.02	0.118±0.04	0.148±0.04
qs (g/g cells/h)	0.567±0.02	0.515±0.02	0.531±0.03	0.504±0.04
Qx (g cells/l/h)	0.063±0.03	0.050±0.01	0.051±0.02	0.040±0.03
	0.054±0.03	0.061±0.03	0.063±0.03	0.074±0.04
Citric acid formation Parameters				
Qp (g/l/h)				
Yp/s (g/g)				
Yp/x (g/g cells)	0.193±0.03	0.449±0.03	0.236±0.03	0.548±0.02
qp (g/g cells/h)	0.340±0.02	0.872±0.03	0.442±0.02	1.087±0.03
	3.600±0.03	7.392±0.04	3.752±0.02	7.360±0.04
	0.021±0.02	0.044±0.02	0.022±0.03	0.043±0.04

Each value is an average of three replicates. ± Indicates standard deviation among replicates. The numbers differ significantly by $p<0.05$. Yx/s = g cells/g substrate utilized, Qs = g substrate consumed/l/h, qs = g substrate consumed/g cells/h, Qx = g cells formed/l/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.

Cultural 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 product formation (Fedoseev, 1970). The apparent viscosity of a culture growing in the pellet form is lower than that corresponding to a filamentous form. Therefore, the quantitative relation between both forms of growth influences the over-all rheological properties of the culture, and hence the effectiveness of mixing and mass transfer (Neilsen, 1992). The positive effect of Cu^{2+} might also be related to the increase of mycelial branching. The presence of shorter and highly branched hyphae probably favours the formation of pellet, improving the performance of the process. It was observed that in the medium without Cu^{2+} (Control, Table I), the mycelial formation stages were not fulfilled. The pellets formed were less (20 pellets /ml) and irregular in form (0.3 - 1.0 mm in diameter). Fluffy loose and round pellets (0.5 mm^2, diameter) were formed by adding 2.0 x 10^{-5}M, $CuSO_4.5H_2O$. Citric acid concentration reached maximum (93.6 ± 5 g/l), 6 hours after the incubation, which was very significant for experiments in stirred fermentor (Table 2). The dry cell mass (16.5 g/l) was slightly higher as compared with wild-culture of *Aspergillus niger*.

The kinetic parameters (Growth yield coefficients, volumetric rates & specific substrate rates) were also studied (Table 3). The mutant strain of *Aspergillus niger* NG-101 significantly improved the values of Yx/s, Yp/s and Yp/x over the parental strain GCB-16. Maximum growth in terms of specific growth rate (μ) was only marginally different during growth of the wild parent and its mutant derivative on 150 g/l carbohydrates in molasses. However, when the cultures were monitored for Yx/s, Qs and qs, there was significant enhancement ($p<0.05$) in these variables with mutant and wild-type culture of *Aspergillus niger*. This indicated that the mutant derivative was a faster growing organism. *Aspergillus niger* mutant exhibited improved production kinetic parameters over the parental strain. Also addition of copper ions (2.0 x 10^{-5}M) in the fermentation medium, 6 hours after incubation provoked the highest values of product yield coefficients as well as volumetric rate constants (Haq *et al.*, 2001; Roehr, 1998; Rajoka *et al.*, 1998). The study revealed that the addition of copper ions have a direct influence on mycelial morphology and pellet formation which increased volumetric productivity of citric acid under liquid culture. It is also hypothesised that the presence of Cu^{2+} ions in the production medium is important in order to enhance a suitable pellet structure with fluffy centre and lax surface and subsequent citric acid production.

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