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OPTIMIZATION OF CITRIC ACID FERMENTATION BY *ASPERGILLUS NIGER* USING CHEMICALLY PRETREATED BLACKSTRAP SUGARCANE MOLASSES

S. Ashraf[1]*, S. Ali[2] and I. Haq[2]

[1]Biology Dept., Govt. Apwa College for Women, Jail Road, Lahore, Pakistan
[2]Institute of Industrial Biotechnology, GC University Lahore, Pakistan
*Corresponding author: shaziashraf1976@yahoo.com

ABSTRACT

The present study deals with the pre-treatment of sugar cane molasses for the enhanced production of citric acid by *Aspergillus niger* NG-4. The maximum production of citric acid (36.4 g/l) was achieved in the medium pre-treated with 250 ppm potassium ferrocyanide. In a parallel study, the acids and metal complexing agents were added in the molasses medium, alternatively prior to heating at 90°C for 1 h. Sugar concentration (150 g/l), intial pH (6) and incubation period (168 h) were also optimized. The kinetic parameters such as growth yield coefficients ($Y_{p/s}$, $Y_{p/x}$, $Y_{x/s}$ in g/g), volumetric rates (Q_p, Q_s, Q_x in g/l/h) and specific substrate rates (q_p, q_s in g/g cells/h) of the research work were also undertaken. The value of Qp (0.134 g/l/h) is highly encouraging ($p \leq 0.05$).

Keywords: Citric acid – *Aspergillus niger* – Microbial fermentation – Sugarcane molasses – Chemical pre-treatment

INTRODUCTION

Citric acid is one of the most important bulk-produced organic acids. It is non-toxic and easily oxidized in the human body [1]. It has been produced on industrial scale by the fermentation of carbohydrates, initially exclusively by *A. niger* but in recent times many microorganisms have been evaluated for the citric acid production including *A. awamori, A. foetidus*, *Penicillium restrictum, Trichoderma viride* and *Mucor pyriformis*. However, *Aspergillus niger*, a filamentous fungus remained the organism of choice for citric acid production [2]. The morphology of filamentous microorganisms during citric acid fermentation varies from round pellets to free long filaments depending upon the cultural conditions and strain genotype. All growth forms have their own characteristics regarding growth kinetics, nutrient consumption and broth toxicity. Although surface culturing is still being used, most of the newly built citric acid plants have adopted submerged fermentation, a more sophisticated technology. The use of beet and cane molasses have made citric acid process economical [3]. Molasses is a

widely used substrate coming in a variety of qualities. The black strap molasses is molasses from the sugar factory obtained from the last stage of crystallization, while refinery molasses is that molasses obtained at a second stage of refining sugar. Potassium ferrocynaide and other complex compounds are commonly used [4].

Major elements such as nitrogen, phosphorus, sulphur and potassium in addition to carbon and various trace elements are needed for the growth of organisms and subsequent citric acid production [5]. Being the basal part of cell proteins, the nitrogen concentration has a profound effect on citric acid yields. On the basis of the present knowledge of *A. niger* metabolism during citric acid fermentation, an idea on how to improve the process was formed. Initially, a higher sucrose concentration was used for the germination of spores, which caused a higher intracellular level of the osmoregulator, glycerol, to be present. When citric acid started to be excreted into the medium, the substrate was suddenly diluted. *A. niger* possesses a pathway of glucose metabolism which is catalyzed by the enzyme glucose oxidase [6]. The present studies are concerned with the pretreatment of cane molasses for citric acid production by *A. niger* NG-4. All the optimizations for citric acid fermentation were carried out in 250 ml shake flasks.

MATERIALS AND METHODS

Organism

A. niger strain NG-4 was obtained from the stock culture of *Biotechnology Research Centre, Department of Botany, GC University Lahore*. It was maintained on potato dextrose agar slants and stored at 4°C in a lab cool (SANYO, Japan).

Molasses clarification

Cane molasses obtained from Kamalia Sugar Mills, Pvt. Ltd. (Kamalia, Pakistan) was pre-treated by acids, metal complexing agents such as potassium ferrocyanide [$K_4Fe(CN)_6$] and ethylene diamine tetra acetic acid (EDTA). The alternative treatment [$K_4Fe(CN)_6$/EDTA] was also undertaken.

Preparation of conidial inoculum

Ten millilitre of sterilized 0.005 % (w/v) diocetyl ester of sodium sulfo succinic acid (Monoxal O.T.) was added to a 3-5 day old slant culture having profuse conidial growth on its surface. The tube was shaken vigorously to obtain a homogenous mixture of the conidial suspension.

Fermentation technique

Twenty-five millilitre of the clarified cane molasses medium containing 150 g/l sugar at pH 6 was added into individual 250 ml cotton plugged conical flasks. The flasks were autoclaved at 15 lbs/in^2 pressure for 15 min. After cooling at room temperature, the flasks were inoculated with 1 ml of the conidial suspension and incubated at 30ºC in a rotary shaking incubator (10X400.XX2.C, SANYO Gallenkamp, PLC, UK) at 200 rpm for 168 h. All the batch culture fermentations were parallel in triplicates.

Analytical techniques

Sugar estimation

The estimation of total reducing sugars (as glucose) is based on the dinitrosalicylic acid (DNS) method [4]. A double beam UV/VIS-scanning spectrophotometer (Cecil-CE 7200-series, Aquarius, UK) was used for measuring the % transmittance. The sugar concentration in culture filtrate was estimated by diluting the filtrate a hundred times. Two millilitres each of the DNS reagent and dilute culture filtrate were added into a test tube. The tube was placed in a boiling water bath for 5 min. After cooling the contents of test tube at room temperature, the mixture was diluted to 20 ml with distilled water. A blank was run in parallel replacing 2 ml of the dilute filtrate sample with distilled water. The % transmittance was estimated at 546 nm on a spectrophotometer.

Estimation of dry cell mass

The dry cell mass was determined by filtering the culture broth through a pre-weighed Whattman filter paper No. 44. Mycelia were thoroughly washed with tap water and dried in an oven (1442A, Memmert, Germany) at 105ºC for 2 h. The filtrate was used for further analysis. The mycelial morphology was determined on an aliquot extended on the petri plates followed by the pellet diameter.

Estimation of citric acid

Citric acid was estimated gravimetrically following the recommended pyridine-acetic anhydride method. The diluted culture filtrate (1 ml) along with 1.3 ml of pyridine was added into a test tube and swirled briskly prior to 5.7 ml of acetic anhydride addition. The test tube was placed in a water bath at 32±0.5ºC for 30 min. The optical density was measured at 405 nm using a spectrophotometer. The citric acid concentration of the sample was estimated from the reference.

Kinetic parameters

The kinetic parameters of batch fermentation process were determined [7].

Statistical analysis

Treatment effects were compared by the protected least significant difference method and one-way ANOVA [8].

RESULTS

Effect of different sugar concentration

The data of Table 1 show the effect of different sugar concentrations such as 90, 105, 120, 135, 150, 165 and 180 g/l on citric acid fermentation by a mutant strain of *A. niger* NG-4 in shake flask. The maximum production of citric acid (66.15 g/l) was observed in the medium containing 150 g/l, initial sugar concentration. The sugar consumption and dry cell mass were 118.88 and 14.12 g/l, respectively. The mycelial growth in the medium was in the form of small pellets and some fussy mass resulting in better agitation. Further increase in citric acid production resulted in gradual reduction in citric acid production.

Rate of citric acid fermentation

The rate of citric acid fermentation by a strain of *A. niger* NG-4 was investigated in shake flask (Table 2). The fermentation was carried out from 24-242 h. After 24 h of incubation, the amount of citric acid produced was 10.5 g/l. Further increase in the incubation period resulted in

increased citric acid production. However, maximum production (57 g/l.) was achieved, 168 h, after inoculation. The sugar consumption and dry cell mass were 93.5 and 14.58 g/l, respectively. The mycelial morphology was mixed mycelium. Further increase in incubation period did not show any enhancement in citric acid production.

Effect of different initial pH

Effect of changing initial pH of the basal medium on the production of citric acid, sugar consumption and dry cell mass by mould culture was investigated (Table 3). Fermentation medium with initial pH 6 resulted in maximum citric acid production (65.2 g/l). The sugar consumption and mycelial dry weights were 93.5 and 16 g/l, respectively. Mixed pellets were observed in the fermented broth. When the pH was increased beyond 6, the production of citric acid was decreased gradually.

Effect of addition of different concentrations of potassium ferrocyanide

Effect of addition of different concentrations of potassium ferrocyanide (50-300 ppm) on citric acid fermentation by a strain of *A. niger* NG-4 was investigated in shake flask (Table 4). The fermentation medium containing 200 ppm potassium ferrocyanide showed the maximum citric acid production (69.3 g/l). The sugar consumption and dry cell mass were 83.5 and 25.3 g/l, respectively. A decrease in citric acid production was observed, when the concentration of potassium ferrocyanide was increased or decreased from 200 ppm.

Kinetic study

Different kinetic parameters such as product and growth yield coefficients (Y p/s, Y p/x, Y x/s), volumetric rates (Qp, Qs, Qx) and specific rate constants (qp, qs) were also studied (Figure 1-3). The values for Yp/s, Yp/x, Qp and qp were more significant after 144 h of incubation than all other time periods, for citric acid production.

DISCUSSION

In the present study, the culture produced maximum citric acid (66.15 g/l) in the medium containing 150 g/l sugars. Further increase in concentration of sugar resulted in the gradual reduction of citric acid formation. It might be due to over growth of the mycelium, which resulted in increased viscosity and mass transfer limitations. A concentration higher than 15-18 % (w/v), however, leads to greater amount of residual sugars, making the process uneconomical, while on the other hand a lower concentration of sugar leads to lower yield of citric acid due to accumulation of oxalic acid. The initial sugar concentration plays an important role in determining the amount of citric acid and also other organic acids produced by *A. niger*. The maximum yield of citric acid (57 g/l) was achieved, 168 h after incubation. Further increase in incubation period did not enhance citric acid production. It might be due to decrease in amount of available nitrogen in fermentation medium, the age of fungi, the presence of inhibitors produced by fungi itself and the depletion of sugar contents. In batch-wise fermentation of citric acid, the production starts after a lag phase of one day and reaches maximum at the onset of stationery phase [9].

Effect of different initial pH of molasses medium of citric acid production was studied and maximum yield (65.2 g/l) was obtained when initial pH of the fermentation medium was 6. Any increase or decrease in the pH greatly reduced citric acid biosynthesis. It might be due to that at lower pH; the ferrocyanide was more toxic for growth of mycelium in molasses medium

[10]. A higher pH leads accumulation of oxalic acid. Thus, the initial pH of basal medium is essential for the successful fermentation of citric acid. The effect of different concentrations of $K_4Fe(CN)_6$ on citric acid fermentation by a mutant strain of *A. niger* NG-110 was carried out in shake flask. The addition of $K_4Fe(CN)_6$ (200 ppm) at the time of inoculation when the medium was hot increased the citric acid production. The insoluble complexes of ferrocyanide with heavy metals acted as metal buffers in the fermentation medium, which made the metal ions available at concentration suitable for citric acid production. It was also due to the fact that it checked the mycelial growth and also inhibited the activity of enzyme aconitase [11]. Further increase in concentration of $K_4Fe(CN)_6$ resulted in the decreased citric acid accumulation. It might be due to toxic inhibitory effect of $K_4Fe(CN)_6$ on fungal growth and decreased sugar consumption.

The kinetic parameters such as growth yield coefficients ($Y_{p/s}$, $Y_{p/x}$, $Y_{x/s}$ in g/g), volumetric rates (Q_p, Q_s, Q_x in g/l/h) and specific substrate rates (q_p, q_s in g/g cells/h) of the research work were also undertaken. The mutant strain of *A. niger* NG-110 showed improved values for $Y_{p/s}$, $Y_{p/x}$, and $Y_{x/s}$ [7, 12]. Maximum growth in terms of specific growth rate (μ in h^{-1}) was only marginally different during growth of mutant *A. niger* GCB-47 on 150 g/l carbohydrates in molasses at 30°C (than 32°C or 165 g/l sugar). However, when the culture was monitored for $Y_{x/s}$, Q_s and q_s, there was a significant enhancement in these variables at optimal nutritional conditions, i.e. incubation temperature 30°C, initial sugar concentration 150 g/l, methanol 1 %, NH_4NO_3 0.15 %, $CaCl_2$ 2 % K_2HPO_4 0.2 % and an incubation period of 168 h (7 days). This indicated that the mutant strain used in the current studies is a faster growing organism and have the ability to overproduce citric acid without additional replacements [13].

CONCLUSION

From the results, it can be concluded, many factors need to be considered by citric acid producers to obtain the economically favourable process. The design of culture media should base on the qualitative and quantitative requirements of nutrients, the interactions between substrates, the physical conditions and medium stability.

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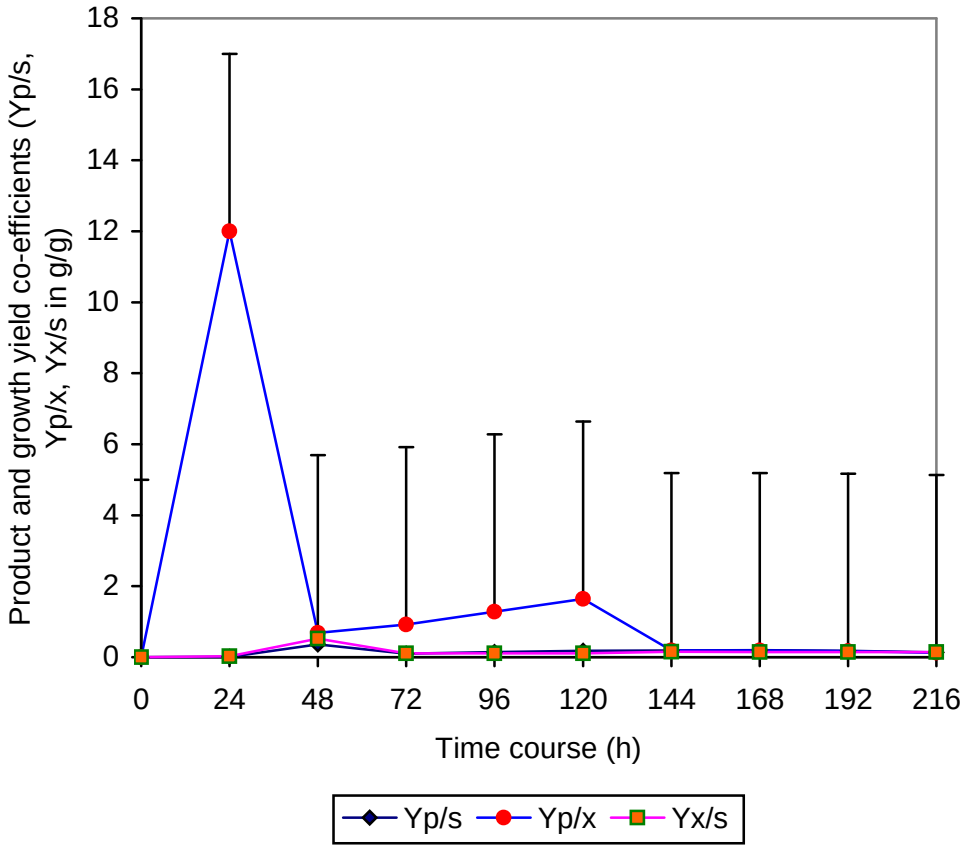

Figure 1. Comparison of product and growth yield coefficients for citric acid fermentation. Yp/s = g citric acid produced /g substrate consumed, Yp/x = g citric acid produced /g cell formed, Yx/s = g cell formed / g substrate consumed. Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

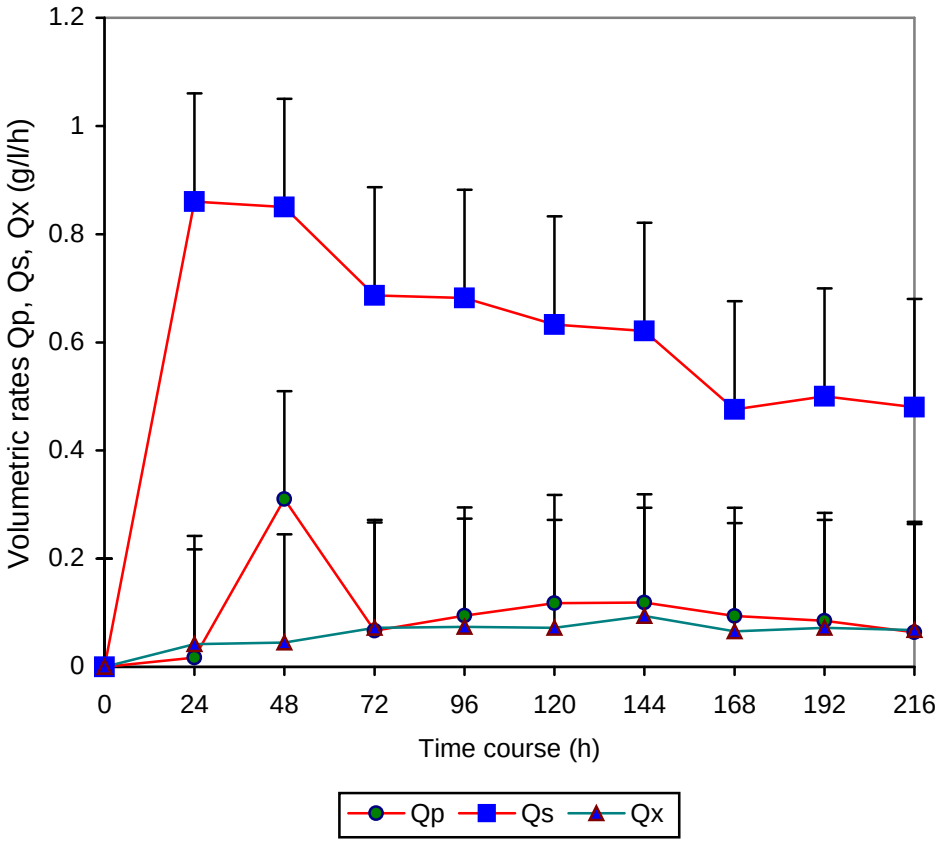

Figure 2. Comparison of volumetric rates for citric acid fermentation. Qp = g citric acid produced /l/h, Qs = g substrate consumed /l/h, Qx = g cell formed /l/h. Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

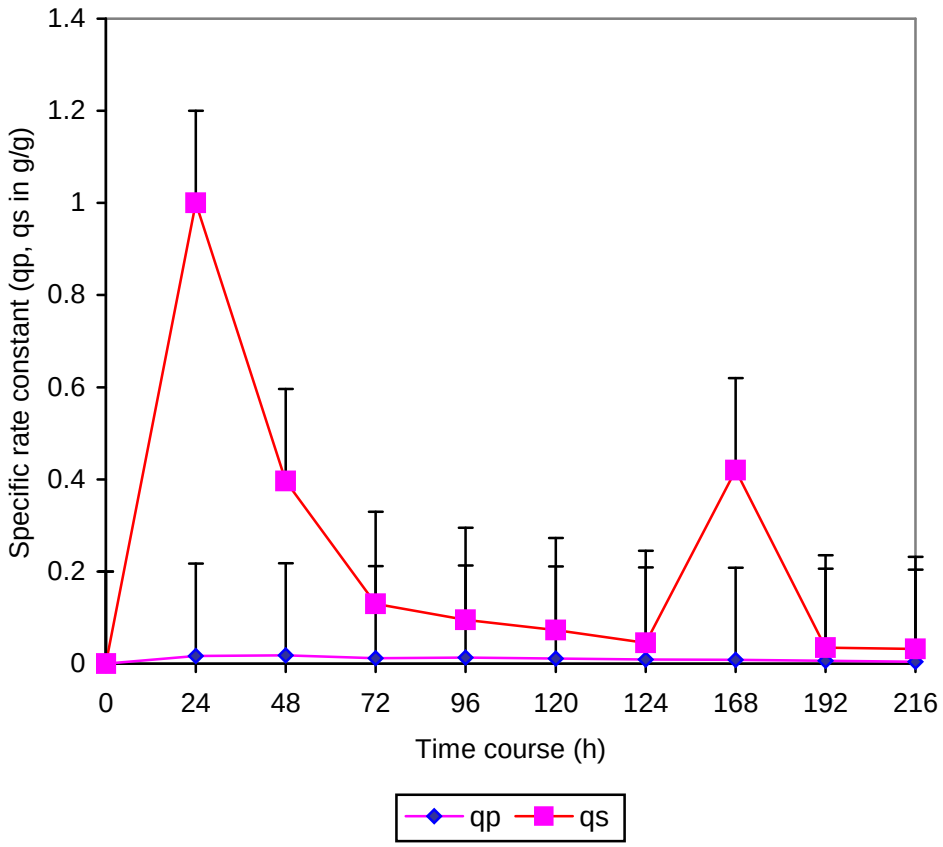

Figure 3. Comparison of specific rate constants for citric acid fermentation. qp = g citric acid produced / g cells/ h, qs = g substrate consumed /g cells/ h. Y error bars indicate the standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Table 1. Effect of different sugar concentration on citric acid fermentation by a mutant strain of *A. niger* NG-4 in shake flask

Sugar conc. (g/l)	Dry cell mass (g/l)	Sugar consumption (g/l)	Citric acid (g/l)	Mycelial Morphology
90	14.36±0.5	79.88±2	35±0.6	Gelatinous
105	14.92±0.8	92.75±1.5	42±0.4	Fine Pellets
120	14.92±0.7	107.25±1.5	49±0.7	Long Fibrous
135	13.16±0.5	119.63±2.5	53±1.2	Broken Fibrous
150	14.12±0.4	118.88±2	66.15±1.1	Pellets & Fussy Mass
165	15.45±0.2	149.75±2	35±1	Pellets & Long Fibers
180	16.23±0.2	161.15±4	30±0.7	Long Fibers

Fermentation period, 168 h; Temperature, 30ºC; Initial pH, 6; Potassium ferrocyanide concentration, 200 ppm. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Table 2. Rate of citric acid fermentation by a mutant strain of *A. niger* NG-4 in shake flask.

Incubation period (h)	Dry cell mass (g/l)	Sugar consumption (g/l)	Citric acid (g/l)	Mycelial morphology
24	6.64±0.2	55±2	10.5±0.1	Elongated mycelium
48	9.5±0.1	70±2.5	21±0.1	Round pellets
72	11±0.2	80.5±2	24.5±0.2	Small round pellets
96	11.58±0.2	85±4	31±0.2	Large round pellets
120	12.32±0.2	91.2±5.5	42±0.2	Small round pellets
144	13.47±0.5	92.3±5	48±0.2	Small round pellets
168	14.58±0.3	93.5±3.5	57±0.4	Mixed mycelium
192	16.79±1.2	99.78±2.9	42±0.5	Gelatinous mass
216	16.2±1	104.48±3.4	35±0.2	Dumpy mass
242	17.98±1.1	110.3±4	39.7±0.4	Dumpy mass

Initial sugar concentration, 150 g/l; Temperature, 30ºC; Initial pH, 6; Potassium ferrocyanide, 200 ppm. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Table 3. Effect of different pH on citric acid fermentation by *A. niger* NG-4 in shake flask

Initial pH	Dry cell mass (g/l)	Sugar consumption (g/l)	Citric acid (g/l)	Mycelial morphology
4.5	9.24±0.8	65.5±3.5	35.±0.4	Mixed
5	12.05±0.7	60.23±3.6	39.5±0.2	Gelatinous mass
5.5	13.35±0.9	98.3±2.8	60.65±0.2	Small pellets
6	16±0.3	93.5±2	65.2±0.2	Mixed pellets
6.5	16.2±0.2	84.2±3	52.5± 0.3	Mixed pellets
7	17.1±0.6	85.75±3.5	45.75±0.2	Gelatinous mass

Initial sugar concentration, 150 g/l; Fermentation period, 168 h; Temperature, 30ºC; Potassium ferrocyanide concentration 200 ppm. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.

Table 4. Effect of addition of different conc. of $K_4Fe(CN)_6$ on citric acid fermentation by a mutant strain of *A. niger* NG-4 in shake flask.

Conc. Of $K_4Fe(CN)_6$ (ppm)	Dry cell mass (g/l)	Sugar consumption (g/l)	Citric acid (g/l)	Mycelial morphology
50	14.75±0.4	57.3±3.5	18.5±0.2	Intermediate size pellets
100	19.2±0.3	72.5±3	27.9±0.4	Gelatinous
150	22.5±0.2	89.75±3.5	52.3±0.6	Small rounded pellets
200	25.3±0.4	83.5±4	69.3±0.8	Mixed Mycelial
250	25.79±0.4	85.45±0.2	65.7±0.4	Small rounded pellets
300	28.4±0.2	92.7±0.2	62.8±0.2	Dumpy mass

Sugar concentration, 150 g/l; Fermentation period, 168 h; Temperature, 30ºC; Initial pH, 6. ± indicates standard error of means among the three parallel replicates. The values differ significantly at $p \leq 0.05$.