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TECHNICAL NOTE

Production of citric acid by *Aspergillus niger* using cane molasses in a stirred fermentor

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Keywords: *Aspergillus niger*, blackstrap molasses, citric acid, fermentation, filamentous fungi, kinetic study.

The present investigation deals with the kinetics of submerged citric acid fermentation by *Aspergillus niger* using blackstrap molasses as the basal fermentation media. A laboratory scale stirred fermentor of 15-L capacity having working volume of 9-L was used for cultivation process and nutritional analysis. Among the 10 stock cultures of *Aspergillus niger*, the strain GCBT7 was found to enhance citric acid production. This strain was subjected to parametric studies. Major effects were caused due to oxygen tension (1.0 l/l/min), pH value (6.0) and incubation temperature (30ºC). All fermentations were carried out following the growth on 150 g/l raw molasses sugars for 144 hours. Ferrocyanide (200 ppm) was used to control the trace metals present in the molasses medium. Ammonium nitrate (0.2%) was added as nitrogen source. Maximum citric acid production (99.56 ± 3.5a g/l) was achieved by *Aspergillus niger* GCBT7. The dry cell mass and sugar consumption were 18.5 and 96.55 g/l, respectively. The mycelia were intermediate round pellets in their morphology. The specific productivity of GCBT7 (qp = 0.074 ± 0.02a g/g cells/h) was several folds higher than other strains. The specific production rate and growth coefficient revealed the hyperproducibility of citric acid using mutant GCBT7.

Citric acid fermentation is one of the rare examples of industrial fermentation technology where academic discoveries have worked in tandem with industrial know-how, in spite of an apparent lack of collaboration, to give rise to an efficient fermentation process. The current world market estimates suggest that upwards of 4.0 x 10^5 tonnes citric acid per year may be produced (Kristiansen et al. 1999). Citric acid is a major product but the upward trend

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in its use seen over many years is an annual 2-3% increase. The demand for this particular metabolite is increasing day by day which requires a much more efficient fermentation process for higher yield product (Moreira et al. 1996). When applied to appropriate mass balances, it is possible to predict the utilization of substrates and the yield of individual products. Fermentation media for citric acid biosynthesis should consist of substrates necessary for the growth of microorganism, primarily the carbon, nitrogen and phosphorus sources. Moreover, water and air can be included as fermentation substrates (Singh et al. 1998; Haq et al. 2001). The basic substrates for citric acid fermentation using submerged technique of fermentation are beet or cane-molasses (Pazouki et al. 2000). The present investigation deals with the kinetic study of citric acid fermentation. Cane-molasses was employed as the basal fermentation media in the stirred fermentor under the submerged fermentation conditions. The study revealed the nutritional status of the organism and basic fermentation parameters.

Materials and Methods

Organism and culture maintenance

Twelve stock cultures of *Aspergillus niger* were obtained from the culture collection of Biotechnology Research Laboratories, Government College, Lahore. These cultures have previously been developed (in our labs) by alternate treatment of ultraviolet irradiations (1.6 x 10^2 J/m^2/S) and nitrosomonas (100 mg/ml) for different time intervals (5-45 min). The cultures of *Aspergillus niger* were maintained on sterilized potato dextrose agar medium (Diced potato 200 g/l, Dextrose 20 g/l and Agar 15 g/l), pH 4.5 and stored at 5ºC in the refrigerator. All the culture media, unless other wise stated, were sterilized in autoclave at 15-lbs/$inch^2$pressure (121ºC) for 15 min.

Pre-treatment of molasses

Cane molasses obtained from different Pakistani Sugar Mills was used in the present study. Cane molasses contains water 20%, sugar contents 62%, non-sugar contents 10%, and inorganic salts (ash contents) 8%, making a blackish homogenous liquid with high viscosity. Ash contents include ions such as Mg, Mn, Al, Fe and Zn in variable ratio (Prescott and Dunn's, 1987). Sugar content was diluted to about 25% sugar level. The molasses solution, after adding 35 ml of 1N H_2SO_4 per litre, was boiled for half an hour, cooled, neutralized with lime-water (CaO) and was left to stand over night for clarification (Panda et al. 1984). The clear supernatant liquid was diluted to 15% sugar level.

Vegetative procedure

Hundred ml of molasses medium (Sugar 15%, pH 6.0) containing glass beads, in 1-L cotton wool plugged conical flask was sterilized. One ml of conidial suspension (6.5 × 10^6 conidia) from the slant culture was aseptically transferred. The conidial count was made by Haemocytometer Slide Bridge. The flask was then incubated at 30ºC in an incubator shaker at 200 rpm for 24 hours.

Fermentation technique

Stainless steel fermentor of 15 L capacity with working volume of 9-L (60%) was employed for citric acid fermentation. Vegetative inoculum was transferred to the production medium at a level of 5% (v/v). The incubation temperature was kept at 30 ± 1ºC throughout the fermentation period of 144 hours. Agitation speed of the stirrer was 200 rpm while aeration rate was maintained at 1.0-4.0 l/l/min. Sterilized silicone oil was used to control foaming during fermentation.

Estimation methods

'Mycelial dry weight' was determined according to Haq and Daud, 1995. 'Sugar' was estimated colorimetrically by Duboise method (1956). A double beam UV/Vis scanning spectrophotometer (Model: CE Cecil-7200 series, UK) was used for measuring colour intensity. 'Anhydrous citric acid' was estimated using pyridine-acetic anhydride method as reported by Marrier and Boulet, 1958. Kinetics of the research work was studied after Pirt, 1975.

Results and Discussion

Screening of stock-cultures of *Aspergillus niger* and molasses media

Twelve cultures of *Aspergillus niger* (Table 1) were screened for citric acid production, following growth on 150 g/l molasses sugar and incubated at 30ºC for 144 hours (found optimum). Of these cultures, *Aspergillus niger* GCBT7 produced higher citric acid (84.95 ± 4.0 g/l). Dry cell mass and sugar consumption were 20.05 and 91.45 g/l, respectively. Mycelial morphology was in the form of intermediate size round pellets. Three cultures gave 58.14 ± 2.7 - 78.18 ± 3.5 g/l, while four cultures produced 18.86 ± 1.8 - 42.56 ± 2.0 g/l citric acid. The citric acid productivity was greater than the 34 cultures studied by Grewal and Kalra, 1995. Cane-molasses obtained from different Pakistani Sugar Mills was screened for citric acid fermentation using the best culture of *Aspergillus niger* GCBT7 (Table 2). The range of citric acid produced was 34.68 ± 2.0f - 85.56 ± 3.5a g/l. The molasses obtained from Kamalia Sugar Mills gave highest yield of citric acid (85.56 ± 3.5a g/l) followed by Premier Sugar Mills (77.65 ± 4.0b g/l). The higher producers of citric acid *i.e.*, GCBT2, 7 and 8 have been compared on the basis of citric acid formation parameters [Qp (g/l/h), Yp/s (g/g), Yp/x (g/g) and qp (g/g cells/h)] and substrate consumption parameters [μ (h^{-1}), Yx/s (g/g), Qs (g/l/h), Qx (g/g cells/h) and qs (g/g cells/h)]. The values of specific rate constants (Qp, Qs and Qx in g/l/h) are more significant in case of *Aspergillus niger*

culture GCBT7 over the other cultures (Table 3). The specific growth and product yield coefficients of GCBT7 *i.e.*, Yx/s = 0.219 ± 0.03b, Yp/s = 0.763 ± 0.05b and Yp/x = 5.229 ± 1.20a g/g are highly significant. In the present study, the specific growth rate (μ = 0.540 ± 0.03b h^{-1}) of GCBT7 is several folds higher as compared with the studies of Pirt, 1975 and Rajoka et al. 1998.

Nitrogen limitation

Nitrogen constituent has a profound effect on citric acid production because nitrogen is not only important for metabolic rates in the cells but it is also the basic part of cell proteins. Effect of different concentrations of ammonium nitrate (as nitrogen source for mycelial growth) on citric acid productivity by *Aspergillus niger* GCBT7 is shown in Figure 1. The maximum amount of citric acid (89.64 ± 1.5a g/l) was obtained when the concentration of NH_4NO_3 was kept at 0.2%. Any increase or decrease other than this concentration, resulted in the disturbance of fungal growth and subsequently citric acid production. The growth rate constant (μ = 0.548 ± 0.02a g^{-1}) indicated that enzyme to substrate ratio was optimum at 0.2% NH_4NO_3. Kristiansen and Sinclair, 1979 used continuous culture and concluded that nitrogen limitation is necessary for citric acid production. Pellet formation in filamentous fungi has been discussed in many cases and among the factors considered to induce it, is the limitation of particular nutrients, including nitrogen. In the present study, the highest values of kinetic parameters *i.e.*, Yp/s = 0.908 ± 0.05a g/g, Qp = 0.618 ± 0.02a g/l/h and qs = 0.036 ± 0.01b g/g cells/h were observed at 0.2% NH_4NO_3.

Rate of citric acid fermentation

The optimal time of incubation for maximum citric acid production varies both with the organism and fermentation conditions. The rate of citric acid biosynthesis was studied (Figure 2a) and the maximum yield of citric acid (94.93 ± 4.2a g/l) was achieved, 144 hours after inoculation. In batch-wise fermentation of citric acid, the production starts after a lag phase of one day and reached maximum at the onset of stationary phase or late. The sugar consumption and dry mycelial weight were 92.94 and 16.15 g/l, respectively. Further increase in incubation period did not enhance citric acid production. It might be due to the decreased available nitrogen in fermentation medium, the age of fungi and depletion of sugar contents. Similar type of work has also been reported by Wieczorek and Brauer, 1998. The kinetics of citric acid production was studied using cultures of *Aspergillus niger* GCBT2 and GCBT7 and the results have been shown in Figure 2b. The product formation rate of GCBT7 (Qp = 0.659 ± 0.03a g/l/h) was 1.46 folds higher as compared with GCBT2 (Qp = 0.417 ± 0.05c g/l/h). Rajoka et al. 1998 obtained 0.0506 ± 0.06 g/l/h product formation rate, which is 1.58 times lower than the present results.

Incubation temperature

The temperature of fermentation medium is one of the critical factors that have a profound effect on the production of citric acid. A temperature of 30ºC was found to be the best for citric acid fermentation (Qp = 0.667 ± 0.02a g/l/h) in present studies (Figure 3). When the temperature of medium was low, the enzyme activity was also low, giving no impact on the citric acid production (Yp/s = 0.444 ± 0.08ef g/g at 24ºC). But when the temperature of medium was increased above 30ºC, the biosynthesis of citric acid was decreased (Yp/s = 0.528 ± 0.06d at 36ºC). It might be due to the accumulation of by-products such as oxalic acid. The value of specific product formation *i.e.*, Yp/x = 6.020 ± 0.02a g/g by *Aspergillus niger* GCBT7 is highly significant. Different workers have also used 30ºC as the cultivation temperatures and obtained higher values of actual product (Vergano et al. 1996; Arzumanov et al. 2000). But when values were divided by the time of fermentation, all values were lower than the one supported by the isolate used in these studies.

Initial pH of fermentation medium

The maintenance of a favourable pH is very essential for the successful production of citric acid. Effect of different pH (4.5 - 7.0) on the citric acid production was studied and maximum yield (96.12 ± 3.5a g/l, anhydrous citric acid) was obtained when initial pH of the fermentation medium was kept at 6.0 (Figure 4). Decrease in pH caused reduction in citric acid production (Qp = 0.319 ± 0.03f g/l/h). It might be due to that at low pH, the ferrocyanide ions were more toxic for the growth of mycelium. This finding is an agreement with Pessoa et al. 1982. A higher initial pH leads to the accumulation of oxalic acid. In fact, a low pH in cane molasses medium has been found inhibitory for the growth of *A. niger* (Yp/x = 3.286 ± 0.04e g/l). The highest value of product yield coefficient *i.e.*, Yp/s = 1.010 ± 0.05a g/g at initial pH 6.0 was much improved by culture GCBT7.

Inoculum size

Among the factors that determine morphology and the general course of fungal fermentations, the type and size of inoculum is of prime importance. In the present study, Figure 5 shows the effect of vegetative inoculum size (0.5 - 3.5%) on citric acid production by *Aspergillus niger* GCBT7 in stirred fermentor. Maximum citric acid production (96.86 ± 4.0a g/l) was obtained with 1.0% inoculum size. All the kinetic parameters *i.e.*, cells yield coefficient (Yx/s = 0.153 ± 0.03g g/g), product formation rate (Qp = 0.507 ± 0.04d g/l/h), volumetric rate of substrate consumption (Qs = 0.680 ± 0.02c g/l/h) and specific rate constant for product formation (qp = 0.034 ± 0.007c g/g cells/h) showed 1.0% vegetative inoculum to be adequate for optimal production of citric acid. This is in accordance with the findings of Van-Suijdam et al. 1980.

Ferrocyanide ion concentration

In the present investigation, the effect of different concentrations of ferrocyanide on the production of citric

acid by *Aspergillus niger* GCBT7 was carried out and their kinetic relations have been shown in Figure 6. The addition of ferrocyanide was made, 24 hours after the inoculation. Maximum citric acid yield (98.28 ± 4.5a g/l, anhydrous citric acid) was obtained at 200 ppm. Further increase in the concentration of ferrocyanide, both the citric acid and the amount of residual sugars were decreased. The amount of mycelial dry weight was continuously decreased by increasing the concentration of ferrocyanide beyond 200 ppm. The specific product formation rate *i.e.*, Yp/x (4.889 ± 0.03d g/g) is highly significant and is 2.56 folds improved as compared with Pirt, 1975. Similarly, the values of growth yield coefficient (Yx/s = 0.191 ± 0.05c g/g) and product formation rate (Qp = 0.611 ± 0.04b g/l/h) indicated higher yields of the product and lower substrate consumption rates. The work is substantiated with the findings of Rajoka et al. 1998.

Concluding Remarks

The culture of *Aspergillus niger* GCBT7 was selected as the best mould to support maximum production of citric acid without supplements. The observation indicates that it might be possible to manipulate the morphology parameters in order to improve bioreactor performance and process yields. Substrate requirement as well as biomass and product yields are some of the basic parameters that need to be considered in determining the feasibility of the fermentation process. All the kinetic parameters *i.e.*, product and growth yield coefficients (Yp/s, Yp/x and Yx/s in g/g), volumetric rates (Qp, Qs and Qx in g/g cells/h) and specific rate constants (qp, qs and qx in g/g/h) are highly significant.

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APPENDIX

Tables

Table 1. Screening of stock cultures for citric acid production. All the fermentations were carried out at 30ºC 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.

Stock cultures of *Aspergillus niger*	Dry cell mass (g/l)	Sugar consumed (g/l)	Citric acid monohydrate (g/l)	% Yield*	Mycelial morphology
GCBT1	16.53	94.65	42.56	44.96	Small shiny pellets
GCBT2	14.95	102.40	78.18	76.35	Intermediate pellets
GCBT3	18.24	78.04	15.25	19.54	Gelatinous mass
GCBT4	16.25	81.52	6.62	8.12	Viscous
GCBT5	23.72	67.82	27.69	40.83	Dumpy mass
GCBT6	14.75	87.64	11.04	12.60	Viscous
GCBT7	20.05	91.45	84.95	92.89	Intermediate pellets
GCBT8	19.12	97.60	72.98	74.77	Mixed pellets
GCBT9	20.14	90.00	18.86	20.96	Gummy mass
GCBT10	22.68	105.28	58.14	55.22	Small round pellets
GCBT11	18.04	99.06	41.02	41.41	Fluffy mass
GCBT12	19.55	89.95	13.34	14.83	Viscous

* based on the sugar consumed. Molasses was obtained from Madina Sugar Mills.

Table 2. Screening of molasses media obtained from different Pakistani Sugar Mills by *Aspergillus niger* GCBT7. All the fermentations were carried out at 30ºC 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.

Sugar Mills	Citric acid anhydrous (g/l)	% Yield*	Mycelial morphology
Madina Sugar Mills	57.60	61.54	Small round pellets
Pattoki Sugar Mills	46.15	54.87	Fine pellets
Kamalia Sugar Mills	85.56	91.90	Intermediate pellets
Premier Sugar Mills	77.65	88.32	Mixed pellets
Rahwali Sugar Mills	34.68	43.70	Gummy mass
Chunian Sugar Mills	62.55	75.24	Mixed broken mycelia

* based on the sugar consumed.

Table 3. Kinetic parameters for citric acid production from molasses sugars following growth of *Aspergillus niger* strains.

Kinetic parameters	GCBT2	GCBT7	GCBT8
Citric acid formation parameters			
Qp (g/l/h)	0.543	0.590	0.507
Yp/s (g/g)	0.763	0.929	0.748
Yp/x (g/g)	5.229	4.237	3.817
qp (g/g cells/h)	0.036	0.029	0.026
Substrate consumption parameters			
μ (h^{-1})	0.540	0.589	0.506
Yx/s (g/g)	0.219	0.219	0.196
Qs (g/l/h)	0.711	0.635	0.678
Qx (g cells/l/h)	0.104	0.139	0.133
qs (g/g cells/h)	0.047	0.032	0.035

Kinetic parameters:
Qp: g citric acid produced/l/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;
μ(h^{-1}): specific growth rate;
Yx/s: g cells/g substrate utilized;
Qs: g substrate consumed/l/h;
Qx: g cell mass produced/l/h;
Qs: g substrate consumed/g cells/h.

Figures

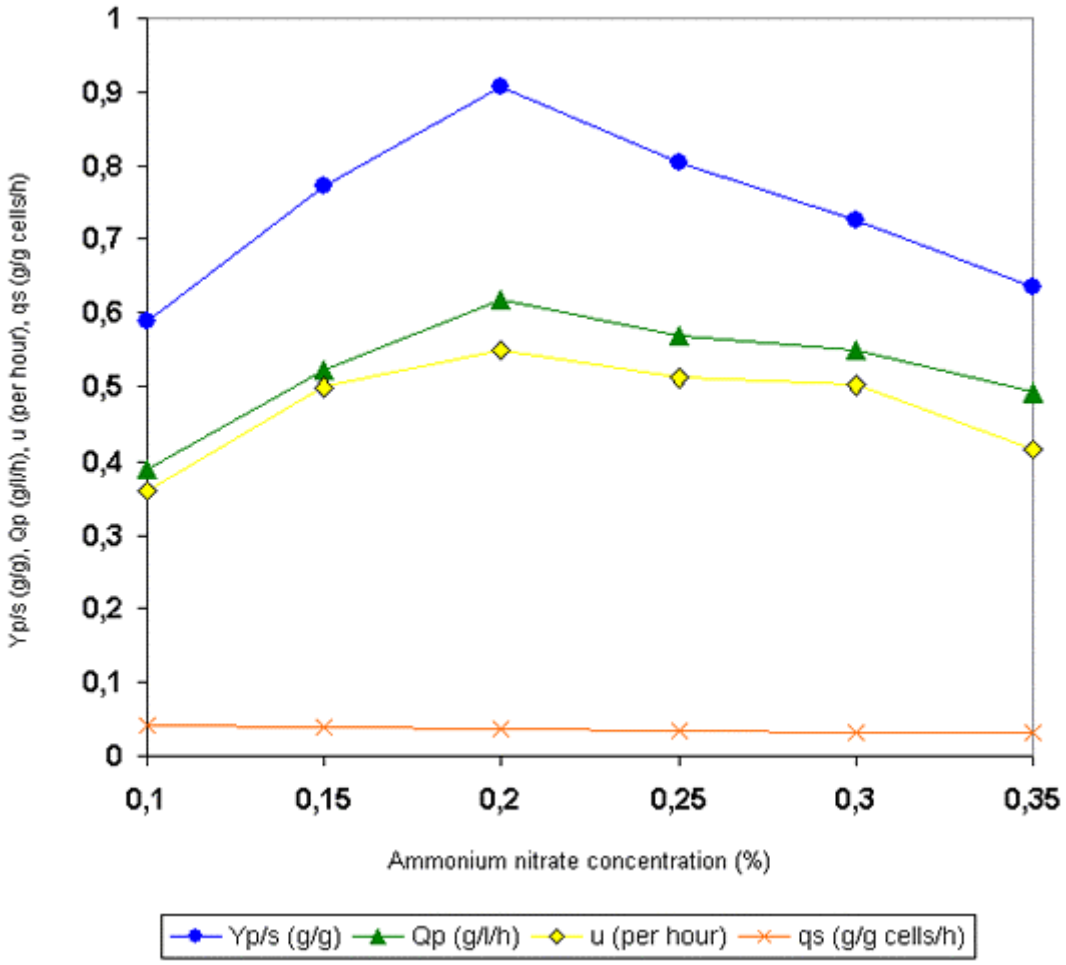

Figure 1. Effect of different concentrations of ammonium nitrate (as nitrogen source for mycelial growth) on citric acid productivity by *Aspergillus niger* GCBT-7. All the fermentations were carried out at 30ºC following growth on 150 g/l initial sugar concentration. The initial pH of the molasses medium was kept constant at 6.0 throughout the fermentation period of 6-days. Yp/s = g citric acid produced/g substrate consumed, Qp = g citric acid produced/l/h, μ (h^{-1}) = specific growth rate, qs = g substrate consumed/g cells/h.

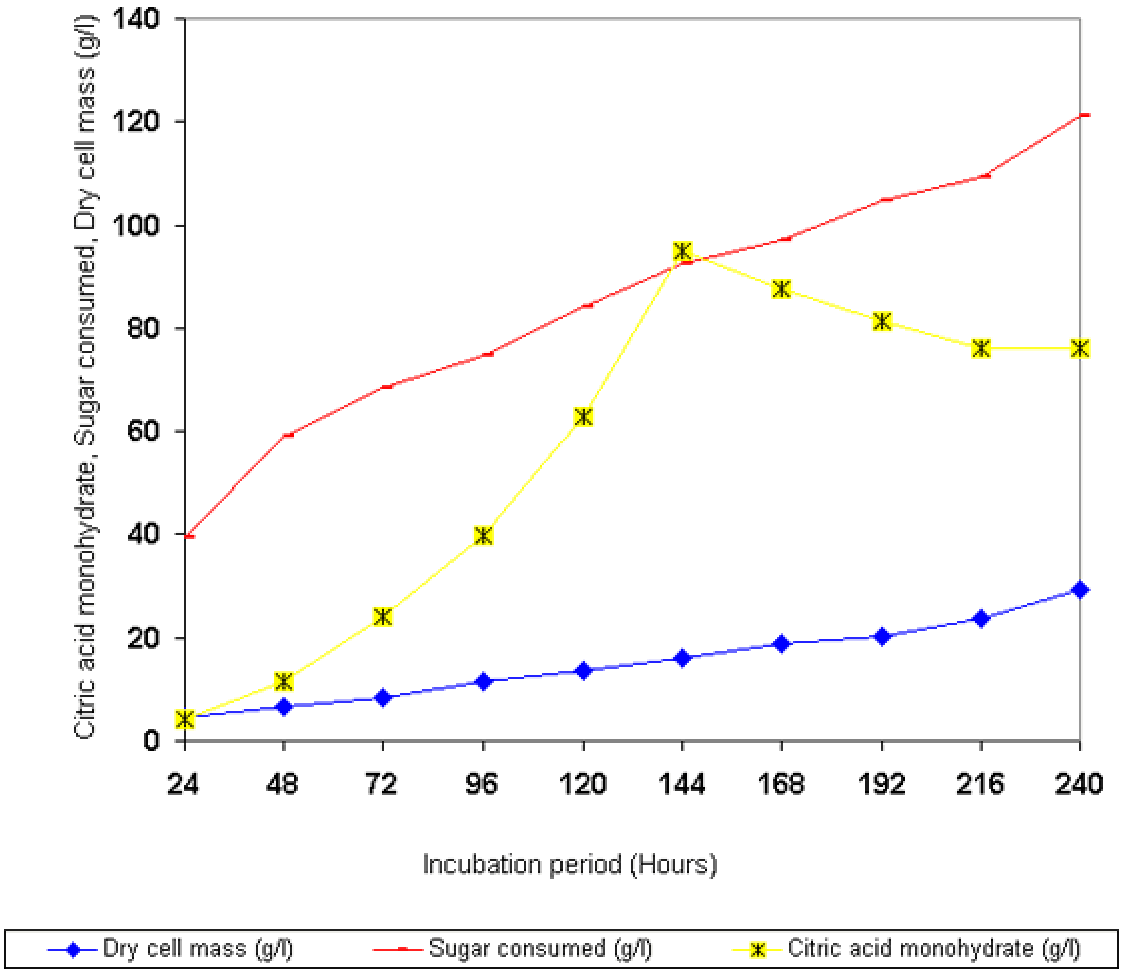

Figure 2a. Time course study during citric acid fermentation by *Aspergillus niger* GCBT7 in blackstrap molasses. All the fermentations were carried out at 30ºC following growth on 150 g/l initial sugar concentration. The initial pH of the molasses medium was kept constant at 6.0.

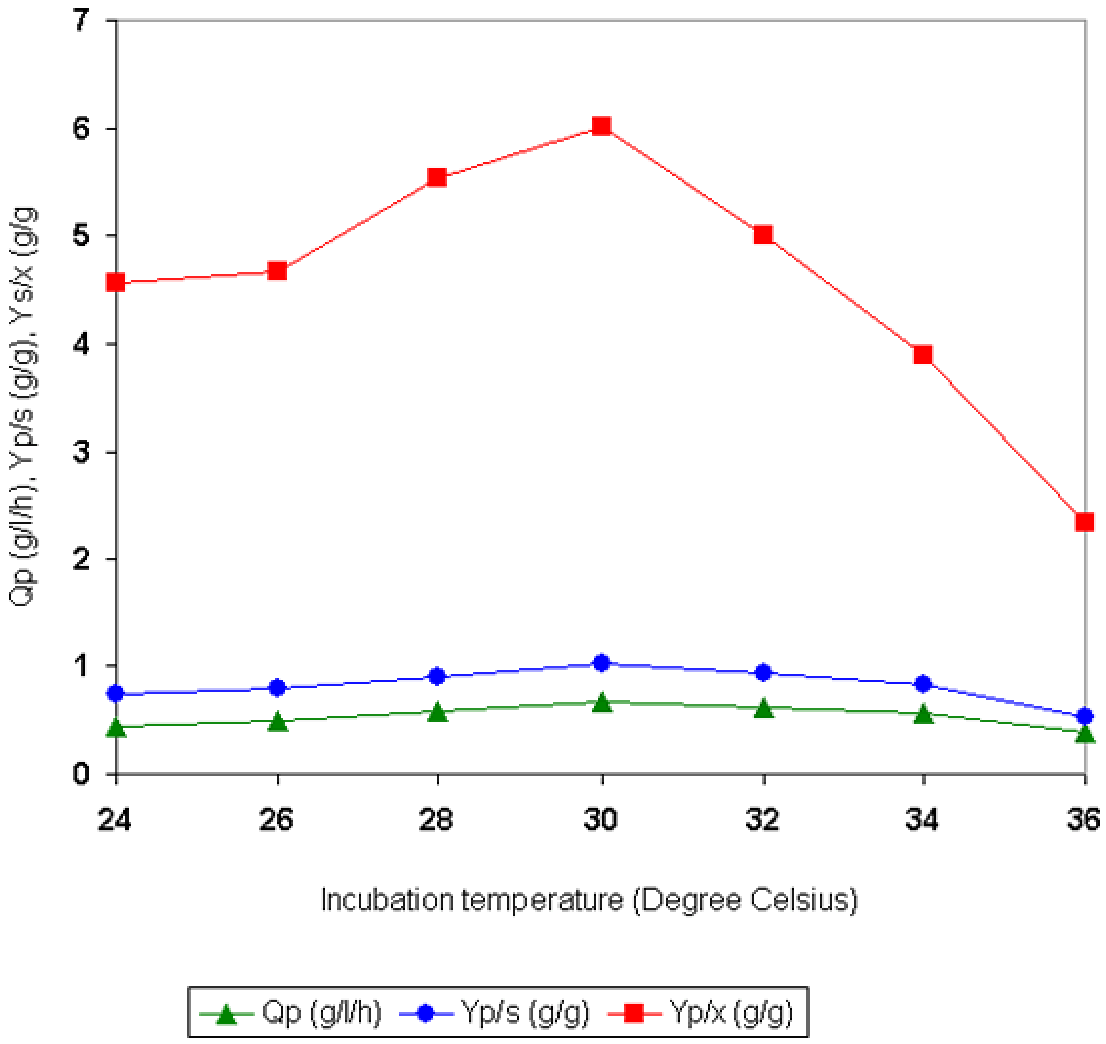

Figure 2b. Comparison of specific product formation rate Qp (g/l/h) during citric acid fermentation by GCBT2 and GCBT7 strains of *Aspergillus niger*. All the fermentations were carried out at 30ºC following growth on 150 g/l initial sugar concentration. The initial pH of the molasses medium was kept constant at 6.0 throughout the fermentation.

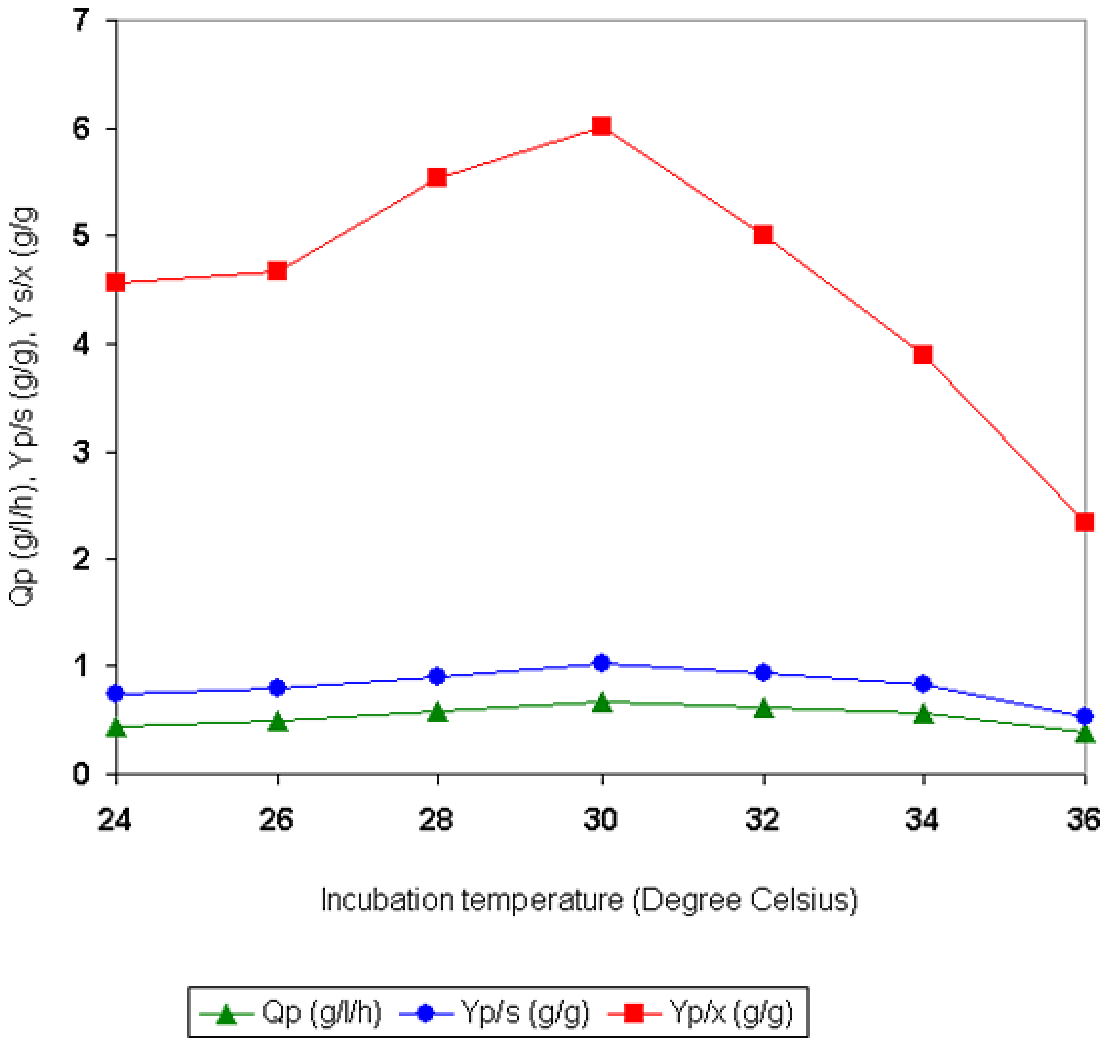

Figure 3. Relative tolerance of temperature for citric acid production by *Aspergillus niger* GCBT7. All the fermentations were carried out at 30ºC following growth on 150 g/l initial sugar concentration. The initial pH of the molasses medium was kept constant at 6.0 throughout the fermentation period of 6-days. Qp = g citric acid produced/l/h, Yp/s = g citric acid produced/g substrate consumed, Yp/x = g citric acid produced/g cells formed.

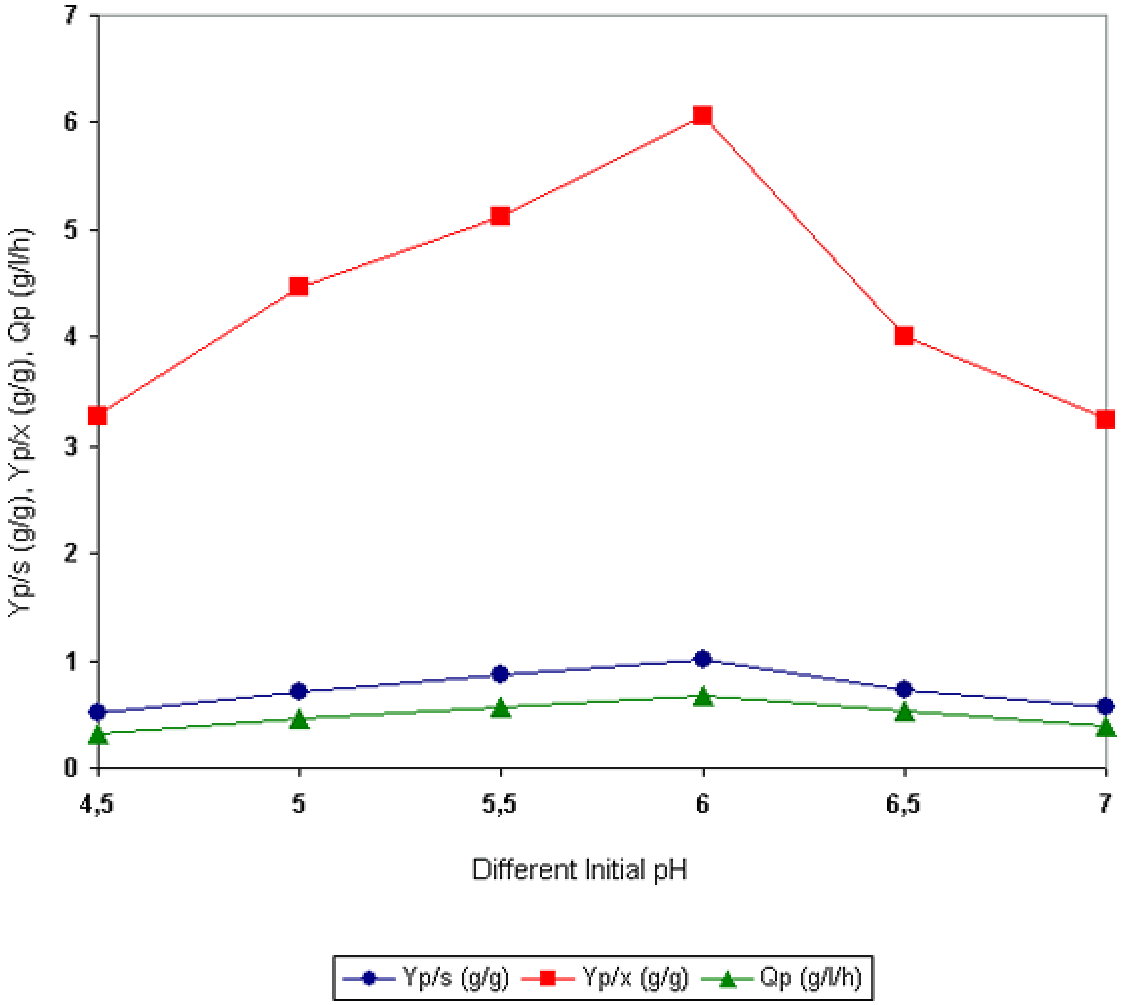

Figure 4. Relative tolerance of initial pH of the fermentation medium for citric acid production by *Aspergillus niger* GCBT7. All the fermentations were carried out at 30ºC following growth on 150 g/l initial sugar concentration. The initial pH of the molasses medium was kept constant at 6.0 throughout the fermentation period of 6-days. Yp/s = g citric acid produced/g substrate consumed, Yp/x = g citric acid produced/g cells formed, Qp = g citric acid produced/l/h.

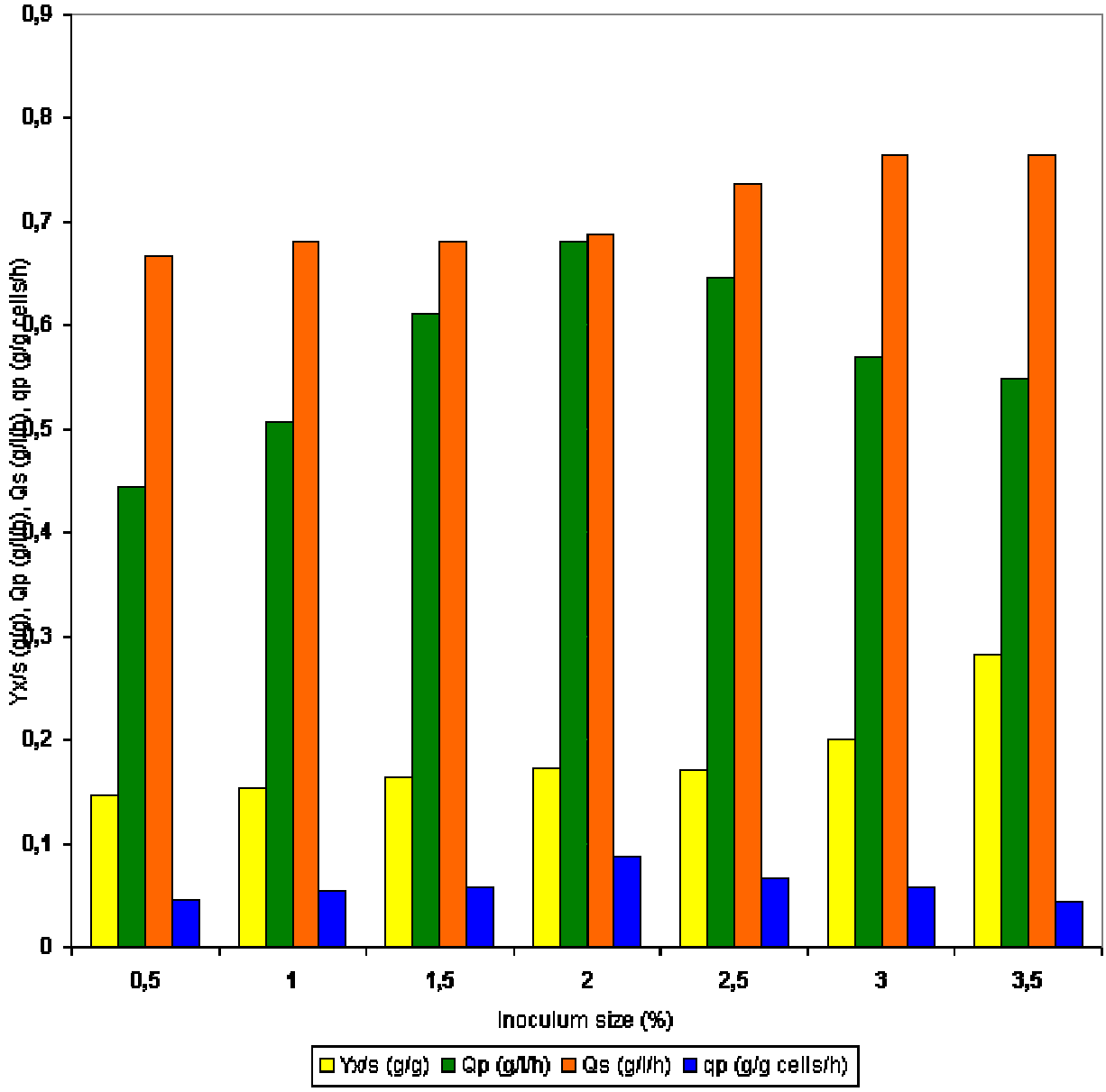

Figure 5. Effect of different inoculum size on citric acid production by *Aspergillus niger* GCBT7. All the fermentations were carried out at 30ºC following growth on 150 g/l initial sugar concentration. The initial pH of the molasses medium was kept constant at 6.0 throughout the fermentation period of 6-days. Yx/s = g cells/g substrate utilized, Qp = g citric acid produced/l/h, Qs = g substrate consumed/l/h, qp = g citric acid produced/g cells/h.

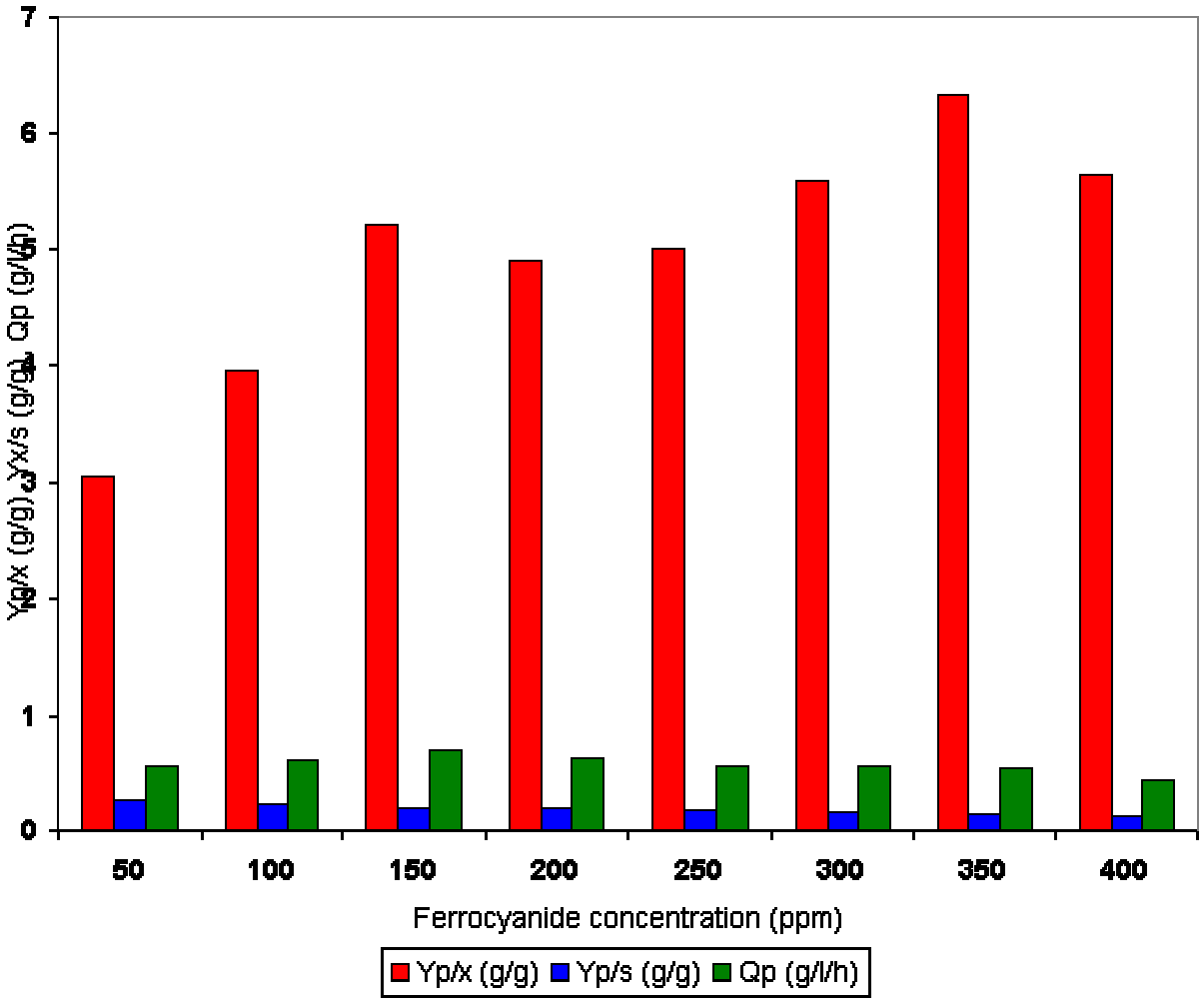

Figure 6. Effect of different Ferrocyanide concentration on citric acid production by *Aspergillus niger* GCBT7. All the fermentations were carried out at 30ºC following growth on 150 g/l initial sugar concentration. The initial pH of the molasses medium was kept constant at 6.0 throughout the fermentation period of 6-days. Qp = g citric acid produced/l/h, Yp/x = g citric acid produced/g cells formed, qp = g citric acid produced/g cells/h.