OnLine Journal of Biological Sciences 1(9): 852-853, 2001

Submerged Fermentation of Citric Acid: Counter-act Effect of Cu^{2+} on the Deleterious Effect of Fe^{2+} in Blackstrap Molasses

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Abstract: Effect of Cu^{2+} were studied ions on the growth morphology and citric acid production from blackstrap molasses by *Aspergillus niger* GCB-47. The addition of 0.02% $CuSO_4$ to the fermentation medium reduced the Fe^{2+} ions conc. by counter-acting its deleterious effect on fungal growth. The Cu^{2+} ions also induced a loose-pelleted form of growth, reduced the biomass concentration and increased the volumetric productivity.

Key words: Citric acid, blackstrap molasses, Cu^{2+} ions, citric acid fermentation, Fe^{2+} ions, reduction in molasses

Introduction

Citric acid fermentation is one of the largest biotechnological industries with a history dating back about a century and most important bulk-produced organic acid (Rajoka *et al.*, 1998). Because of its high solubility and low toxicity, citric acid can be used in foods and pharmaceutical industries (Roehr, 1998). Citric acid is a primary product of *Aspergillus niger* metabolism (Haq *et al.*, 2001). Environmental conditions markedly influence the growth pattern of filamentous fungi, in this way affecting the product formation (Pera and Callieri, 1997).

Citric acid produced by *Aspergillus niger* is extremely sensitive to trace metals present in molasses (Majolli and Aguirre, 1999) the trace elements such as iron, zinc, copper & manganese present a critical problem in submerged fermentation. The concentration of these heavy metals, therefore, should be decreased well below that required for optimal growth as well as maximum citric acid production (Maria and Wladyslaw, 1989). The microorganisms also need major elements such as carbon, nitrogen, phosphorous and sulphur in addition to various trace elements (Khan *et al.*, 1970). In this experiment, attempts have been made to increase the citric acid production by reducing Fe^{2+} concentration in blackstrap molasses.

Materials and Methods

The parent *Aspergillus niger* strain GCB-47 (an isolate from our own culture) was used for citric acid fermentation. The culture was maintained on potato dextrose agar medium, pH 4.5 (Fluka, Switzerland) and the slants were stored at 5°C in the refrigerator.

Fermentation Technique: Vegetative inoculum was used in the present study, which was developed according to the method of Khan *et al.* (1970). Stainless steel fermentor of 15L capacity with working volume of 9L was employed for fermentation. The fermentation medium consisting of; cane-molasses (sugar 15%) $K_4Fe(CN)_6$ 200 ppm having pH 6.0 was sterilized at 15 lbs/inch^2 pressure (121°C) for 15 min. Vegetative inoculum was used at a level of 5% (v/v). The incubation temperature was kept at 30± 1°C throughout the fermentation period of 6 days. Agitation speed and aeration rate were kept at 200 rpm and 1.0 l/l/min, respectively. Sterilized silicone oil was used to control the foaming problem during fermentation.

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. Total acid was determined by titrating 1.0 ml of fermented broth against 0.1N NaOH using phenolphthalein as an indicator.

$$\% \text{ total acid} = \frac{\text{Titre x Normality of alkali x Eq. wt. of acid}}{\text{Volume of sample x 1000}} \times 100$$

Anhydrous citric acid was determined colorimetrically using pyridine-acetic anhydride method (Marrier and Boulet, 1958). The % age of citric acid was determined by using the formula:

$$\% \text{ citric acid} = \frac{\text{Citric acid}}{\text{Sugar used}} \times 100$$

Sugar was estimated by DNS method (Tasun *et al.*, 1970) while. Ferrocyanide concentration was determined colorimetrically (Marrier and Clark, 1962).

Results and Discussion

Effect of different copper sources [$CuCl_2$, $CuSO_4.5H_2O$, $Cu(NO_3)_2$] on the production of citric acid by *Aspergillus niger* GCB-47 in stirred fermentor was showed in Table 1. Among all of these, $CuSO_4.5H_2O$ gave maximum production (81.55 g l^{-1}) of citric acid. It may 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 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 mycelium.

The effect of different concentrations (0.01 - 0.04%) of $CuSO_4$ on the production of citric acid was also carried out (Table 2). At low concentration of Cu^{2+} ions (0.01%), the production of citric acid was also low (70.0 g l^{1}). Maximum amount of anhydrous citric acid (87.65 g l^{1}) was obtained when 0.02% $CuSO_4.5H_2O$ was used as a source of Cu^{2+} ions in the fermentation medium. Mycelia were in the form of small round pellets. The dry mycelial mass was found to be 16.5 g l^{-1}. Sugar consumption was 107 g l^{1} and %age of citric acid on the basis of sugar used was calculated as 82.57%. Final pH and residual $K_4Fe(CN)_6$ concentration were 2.3 and 40 ppm, respectively. The presence of $CuSO_4.5H_2O$ concentration higher than 0.02% did not have any (major) effect on the structure and external aspect of the pellets, but affected citric acid production which remarkably decreased (66.10 g/l, 44.02 g l^{-1}). The results presented show that the pellets with a suitable structure and morphology improve citric acid production. Some authors believed that Cu^{2+} may stimulate citric acid production by inhibiting aconitase (Bruathman, 1961; Majolli and Aguirre, 1999) others reject this possibility,

Table 1: Effect of different copper sources on the production of citric acid by *Aspergillus niger* GCB-47 in stirred fermentor.

Copper sources (0.015%)	Total acid (g l-1)	Anhydrous citric acid (g l^{-1})	Dry cell mass (g l^{-1})	Final pH	Final $K_4Fe(CN)_6$ (ppm)	Sugar (g l^{-1}) Used	Residual	% citric acid on the basis of sugar used	**Mycelial** morphology
Blank	49.10	38.26	21.0	4.2	45	109	41	35.10	Gelatinous
$CuSO_4$. $5H_2O$	86.00	81.55	15.0	2.4	60	104	46	78.80	Lax, fluffy & small pellets
$CuCl_2$	35.85	23.72	13.5	4.5	80	120	30	19.77	Gelatinous
$Cu(NO_3)_2$	56.20	46.08	16.5	3.7	55	114	36	40.42	Irregular shaped pellets

Table 2: Effect of different concentrations of $CuSO_4.5H_2O$ on the production of citric acid by *Aspergillus niger* GCB-47 in stirred fermentor.

CuSO4. 5H2O conc. (%)	Total acid (g l-1)	Anhydrous citric acid (g l^{-1})	Dry cell mass (g l^{-1})	Final pH	Final $K_4Fe(CN)_6$ (ppm)	Sugar (g/l) Used	Residual	% citric acid on the basis of sugar used	**Mycelial** morphology
0.01	71.25	70.00	21.5	3.2	60	122	28	57.38	Small pellets
0.02	89.06	87.65	16.5	2.3	40	107	43	82.57	Fluffy center & lax surface
0.03	68.98	66.10	13.2	3.6	40	128	22	51.64	Mixed pellets
0.04	52.65	44.02	13.0	3.9	45	126	24	34.94	Gelatinous

Cultural conditions: Temperature = 30°C, Aeration rate = 1.0 ll^{-1} min^{-1}, initial sugar concentration = 150 g/l, Initial pH = 6.0

finding no alterations in the citrate-isocitrate ratio when adding Cu^{2+} under the conditions of citric acid production. However they observed favorable changes in citric acid yield as well (Kubiceck and Rohr, 1985).

Environmental conditions markedly influence the growth pattern of filamentous fungi, which can be ranged from a pelleted to a dispersed filamentous form, affecting in this way both the growth rate and product formation. The apparent viscosity of a culture growing in the pelleted from 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 (Nielsen, 1992). The positive effect of Cu^{2+} might also be related to the increase of the mycelial branching level. The presence of shorter and highly branched hyphae probably favours the formation of pellet, improving the performance of the process (Metz and Kossen, 1977; Rajoka *et al.*, 1998). It was observed that in the medium without Cu^{2+} (Blank, Table 1), the mycelial formation stages are not fulfilled. The pellets formed are less (20 pellets /ml), irregular inform (0.3 - 1.0 mm in diameter) with abundant. Filamentous mycelium growth increasing in time. This yielded a very low citric acid content (46.08 g l^{-1}) in the production medium. As desired fluffy loose and 0.5 mm diameter pellets were formed by adding (0.02%) $CuSO_4.5H_2O$. The acid concentration reached (77.65 g l^{-1}) which was very interesting for experiments in stirred fermentor (Table 2). The dry cell mass (16.5 g l^{-1}) was slightly higher in this case.

Aspergillus niger needs a variety of divalent trace elements such as Fe^{++}, Cu^{++}, Zn^{++}, Mg^{++}, etc. for growth and citric acid production. 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 (Schweiger, 1961; Khan *et al.*, 1970) that citric acid production by *A. 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: however, the addition of copper at 0.1 - 500 ppm at the time of inoculation or during the first 50 hours of fermentation was found to counter-act the deleterious effect of iron. Such beneficial effect of Cu^{++} in counteracting the Fe^{2+} effect has also been reported earlier (Fedoseev, 1970) and it was found that the addition of $CuSO_4$ at 4.7 mg/100g molasses resulted in better conversion of sugar into citric acid. It is concluded that the presence of different Cu^{2+} concentrations in the pellet formation medium is very important in order to enhance a suitable pellet structure (fluffy center and lax surface) related to major cellular physiology to citric acid production. The optimum initial $CuSO_4.5H_2O$ concentration in the experiment was 0.02% and citric acid was the only organic acid produced.

Acknowledgements

Pakistan Science Foundation, under the Project PSF/Res/P-GC/Bio (283) funded this work.

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