EFFECT OF FERROCYANIDE ON THE PRODUCTION OF CITRIC ACID FROM CANE MOLASSES BY ASPERGILLUS NIGER

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The citric acid fermentation of cane molasses by *Aspergillus niger* was studied. Addition of ferrocyanide (600 ppm) greatly increased the citric acid yield (60 g/l). The important factors in the production of citric acid are ferrocyanide concentration, morphology of the mould growth and initial pH of the medium. The insoluble complexes of ferrocyanide with heavy metals acted as metal buffers in the fermentation media which made the metal ions available at concentration suitable for maximum citric acid production. The concentration of free ferrocyanide was slightly affected during fermentation. Clarification of the molasses media showed no significant effect on citric acid production.

Introduction

The mineral contents of the molasses particularly trace metals such as iron, zinc, copper and manganese present a critical problem in submerged citric acid fermentation by *Aspergillus niger*.[1-3] Potassium ferrocyanide forms insoluble complexes with heavy metals and thus it has been generally used to reduce the trace metal concentrations of fermentation media which increases the citric acid production.[4-8] In addition to the removal of heavy metals, ferrocyanide has direct toxic effect on the mould metabolism as reported by Martin.[6]

The present investigations describe the effect of ferrocyanide on citric acid production by *Aspergillus niger* from cane molasses, obtained from sugar factories in West Pakistan. The effect of free ferrocyanide and insoluble complexes in the citric acid fermentation has also been studied.

Methods

Mould Strains.—The strains of *Aspergillus niger* used were; wis. 72-4, locally isolated WRL 14, WRL 50 and WRL 51.

Inoculum Preparation.—Spore inoculum was used in the present study. A simple synthetic agar medium—containing (g/l): sucrose, 150; agar, 20; NH_4NO_3, 2.5, KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25, and trace metals (mg/l): Fe++ ($FeCl_3$) 2.20; Cu++ ($CuSO_4$), 0.48 and Zn++ ($ZnSO_4$), 3.80—was used. Inoculum medium was autoclaved at 121° for 15 min. 15 ml of agar medium was transferred in a cotton wool-plugged bottle and the cultures were incubated at 30°. Spores from 5 to 7 day old cultures were wetted with 5 ml of 0.05% solution of sodium lauryl sulphate. The plate was washed with sterile distilled water. The combined washings were made up to 50 ml and shaken with glass beads to break up clumps of spores. 25 ml of fermentation medium in 300 ml flask was inoculated with 1 ml of spore inoculum.

Fermentation Medium.—Cane molasses obtained from Mardan, Charsadda, Rahwali and Lyallpur sugar factories in West Pakistan were used for fermentation. Sugar contents of the molasses were 60, 52, 47 and 43% respectively. For medium preparation, the molasses were diluted to 15% sugar concentration with tap water. The molasses solution was adjusted to pH 6.0 and sterilized at 121° for 15 min. Potassium ferrocyanide solution was sterilized by membrane filter.

Conditions of Cultivations.—The culture temperature was 30°. All fermentations were carried in shake flasks. The rotary shaker was placed in a room at 28±2°. The flasks were rotated at 200 rev/min with 1½ in. amplitude throw.

Molasses Clarification.—The molasses solution, after adding 35 ml. of 1 N H_2SO_4 per litre, was boiled for ½ hr, cooled, neutralized after cooling with lime water and left to stand overnight. The clear supernatant liquid was used for fermentation.

Analytical Methods.—Mycelial dry weight was determined by filtering 25 ml of culture through weighed Whatman paper No. 41, and washed 3-4 times with tap water. The mycelium was dried at 105° overnight before weighing. Citric acid was estimated colorimetrically by the method of Marrier and Boulet[9] and sugars by ferrocyanide reduction method, a modification of Fujita and Watake.[10] Free ferrocyanide was estimated by the method of Marrier and Clark.[11]

Results

Selection of Strain.—Unclarified Rahwali molasses medium was employed for citric acid fermentation. Ferrocyanide addition was made at the

time of spore inoculation. The strain of *Aspergillus niger* Wis. (72–4) produced maximum amount of citric acid (52 g/l). Out of three locally isolated strains of *Aspergillus niger*, WRL 50 gave more citric acid yields (39 g/l) (Table 1). However, it was lesser than that obtained with wisconsin strain. In subsequent experiments, *Aspergillus niger* wis. 72-4 was used for citric acid fermentation.

Citric Acid Fermentation of Unclarified Molasses.—Fig. 1 shows the effect of 0–1200 ppm ferrocyanide, added at the time of inoculation, on the biosynthesis of citric acid in unclarified molasses media. Molasses of Rahwali and Lyallpur sugar mills gave maximum citric acid yield (60 g/l) in the presence of 600 ppm ferrocyanide. Further increase in the concentration of ferrocyanide resulted in lowering the citric acid production. The amount of citric acid produced in Mardan and Charsadda molasses media, however, was 35 g/l) and optimum level of ferrocyanide was 300 ppm.

TABLE 1.—PRODUCTION OF CITRIC ACID IN RAHWALI MOLASSES MEDIA BY DIFFERENT STRAINS OF *Aspergillus niger*.

Strain	Ferrocyanide ppm			
	0	300	600	900
	citric acid g/l			
WRL-14	5	15	20	13
WRL-50	8	19	39	21
WRL-51	4	13	17	12
Wis. 72-4	23	38	52	31

Citric Acid Fermentation of Clarified Molasses.—The effect of ferrocyanide (0–1200 ppm) added at the time of inoculation, on citric acid production, in clarified molasses media, was also studied (Fig. 2). The citric acid production in Rahwali and Lyallpur molasses media remained about the same as that obtained in unclarified molasses media. In Charsadda and Mardan molasses media, however, the production of citric acid was greater (50 g/l) than that produced in unclarified molasses media (35 g/l).

Since maximum citric acid yield of 60 g/l in unclarified molasses media remained almost unchanged by clarification. Unclarified Rahwali molasses medium was used in subsequent experiments.

Effect of Ferrocyanide Addition under Different Conditions on Citric Acid Production.—Citric acid yield of 56 g/l was obtained by adding ferrocyanide at the time of inoculation. The yield was 26 g/l when

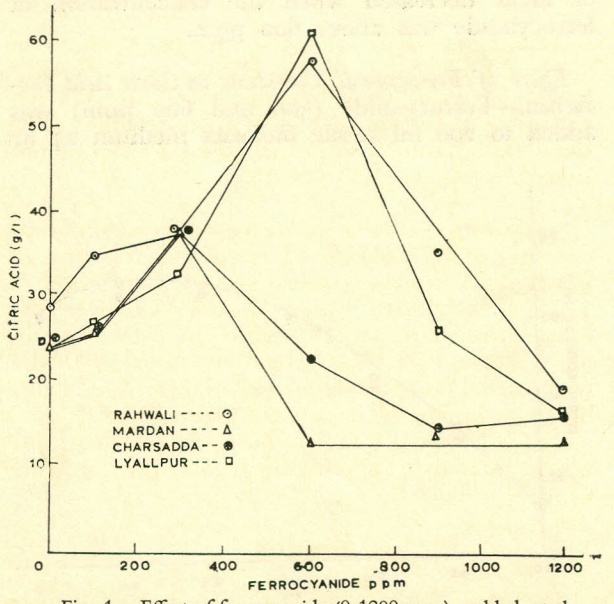

Fig. 1.—Effect of ferrocyanide (0-1200 ppm), added at the time of inoculation, on citric acid production by *A. niger* in unclarified molasses media.

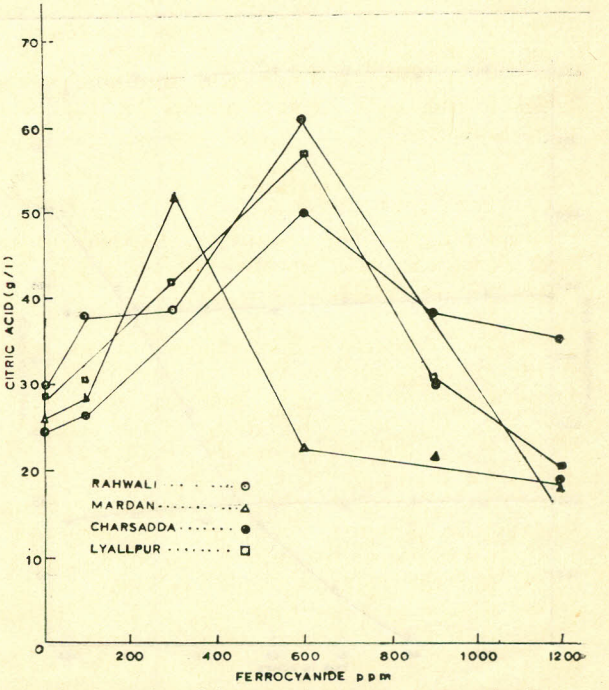

Fig. 2.—Effect of ferrocyanide (0-1200 ppm), added at the time of inoculation, on citric acid production by *A. niger* in clarified molasses media.

ferrocyanide was added to the medium before sterilization. However, when ferrocyanide concentration was maintained at 150–190 ppm, the yield of citric acid further increased to 50 g/l. Addition of ferrocyanide, just after sterilization, gave 40 g/l citric acid (Table 2). The cause of low citric acid production may be due to the decomposition of ferrocyanide at high temperature.

Change in Ferrocyanide Concentration During Fermentation.—The amount of free ferrocyanide in the culture medium during fermentation was also estimated (Fig. 3). Ferrocyanide levels in the medium were 200, 490 and 800 ppm respectively. Citric acid was estimated only in cultures containing 600 ppm ferrocyanide. The concentration of free ferrocyanide in the culture medium decreased about 20–30 ppm during fermentation. Free ferrocyanide concentration of 200 ppm gave maximum citric acid production.

TABLE 2.—EFFECT OF FERROCYANIDE ADDED UNDER DIFFERENT CONDITIONS ON CITRIC ACID YIELD.

	Citric acid yield after 168 hr g/l			
Ferrocyanide conc. ppm	Ferrocyanide added at the time of inoculation	Ferrocyanide added just after sterilization	Ferrocyanide added before sterilization	Ferrocyanide added before sterilization & its conc. maintained between 150-190 ppm
300	40	29	20	38
600	56	36	24	50
900	35	40	26	41

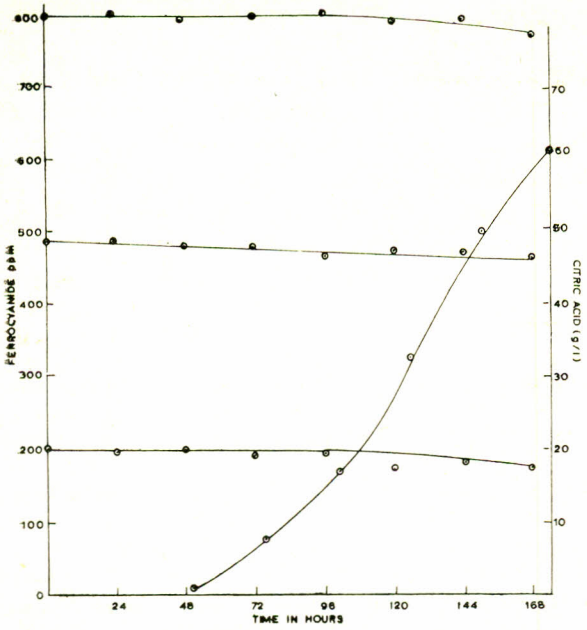

Fig. 3.—Change in ferrocyanide concentration during fermentation.

Effect of pH.—Ferrocyanide (600 ppm) was added at the time of inoculation. The citric acid production was maximum at pH 6.0–6.5 (Fig. 4). Ferrocyanide at low pH seems toxic to the mould resulting in a decrease in citric acid formation. The mould growth was inhibited when ferrocyanide was added at pH 3.0.

Effect of Ferrocyanide on Mould Growth.—The mould growth in control cultures of both clarified and unclarified molasses media was in the form of large filamentous and gelatinous pellets. This affected aeration of the cultures. Addition of ferrocyanide modified the mould morphology and growth was in the form of separate, round and smooth pellets, thus increasing the aeration of the cultures. The size of the pellets as well as mycelial dry weight decreased with the increase in the concentration of ferrocyanide showing its inhibitory effect on the growth process (Table 3). The mycelial dry weight in the clarified molasses was lesser than that in unclarified molasses media. It may be due to the higher concentration of free ferrocyanide in the clarified molasses media due to the removal of heavy metal by clarification.

Table 4 shows the effect of ferrocyanide (0–1200 ppm), added at the time of inoculation, on sugar utilization by *Aspergillus niger*. The utilization of sugar decreased when the concentration of ferrocyanide was above 600 ppm.

Effect of Ferrocyanide Complexes on Citric Acid Production.—Ferrocyanide (300 and 600 ppm) was added to 200 ml sterile molasses medium 24 hr

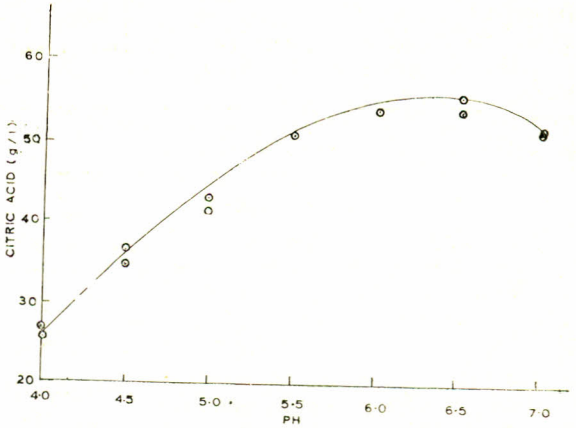

Fig. 4.—Effect of initial pH of the molasses medium on citric acid production by *A. niger*.

TABLE 3.—EFFECT OF FERROCYANIDE ON MYCELIAL DRY WEIGHT.

Ferrocyanide conc. ppm	Dry weight g/l after 168 hr							
	Rahwal		Mardan		Charsadda		Lyallpur	
	Uncl.	Cl.	Uncl.	Cl.	Uncl.	Cl.	Uncl.	Cl.
Blank	33.31	33.64	28.60	17.40	33.80	21.48	32.23	19.16
100	34.28	32.92	28.12	19.84	32.56	29.56	32.04	22.30
300	31.06	30.48	24.52	20.32	32.72	28.76	28.64	23.92
600	20.10	29.28	23.28	19.22	30.20	19.60	28.12	20.84
900	20.38	20.04	15.32	4.48	34.36	19.20	28.72	20.36
1200	19.45	18.08	14.08	4.29	28.88	19.34	26.00	20.52

Uncl., Unclarified; Cl., Clarified.

TABLE 4.—EFFECT OF FERROCYANIDE ON SUGAR UTILIZATION.

Ferrocyanide conc. ppm	Sugar utilized g/l after 168 hr							
	Rahwali		Lyallpur		Charsadda		Mardan	
	Uncl.	Cl.	Uncl.	Cl.	Uncl.	Cl.	Uncl.	Cl.
0	93	95	84	77	60	65	70	75
100	79	102	95	90	62	76	75	75
300	95	100	93	95	80	79	90	100
600	105	110	108	101	60	98	70	76
900	65	75	69	90	65	80	65	46
1200	60	80	71	93	64	76	62	40

Uncl., Unclarified; Cl., Clarified.

before inoculation. Part of the medium was inoculated with spores after removing insoluble ferrocyanide complexes by membrane filtration. Thus cultures with and without ferrocyanide complexes were run parallel (Table 5). The mould growth in cultures containing ferrocyanide complexes was in the form of separate, smooth pellets and citric acid yield was 54 g/l. The mould growth in cultures without ferrocyanide complexes, however, was in the form of large filamentous and gelatinous pellets which resulted in lowering citric acid yield to 25 g/l.

TABLE 5.—EFFECT OF FERROCYANIDE COMPLEXES.

Ferrocyanide conc. ppm	With complexes		Without complexes	
	Citric acid g/l	Dry weight g/l	Citric acid g/l	Dry weight g/l
300	38	29.326	20	30.721
600	54	23.569	25	27.231

Discussion

The metabolic system of *Aspergillus niger* in submerged fermentation is greatly influenced by the addition of ferrocyanide. Important factors in the biosynthesis of citric acid are the morphology of mould growth, initial pH of the medium and precise control of ferrocyanide concentration. The exact mechanism of ferrocyanide action in increasing citric acid biosynthesis is obscure. The insoluble complexes of ferrocyanide with heavy metals act as metal buffer due to their slight solubility at low pH[12] thus providing metal ions, particularly iron, at concentrations suitable for maximum citric acid production.[13] The direct action of ferrocyanide on the metabolism of *Aspergillus niger* may also have an important effect in supressing the enzymes involved in further metabolism of citric acid via the Kreb cycle.[14] Ferrocyanide concentration of 600 ppm was sufficient in both adjusting trace metal constituents of the molasses medium and stimulating citric acid formation. The amount of free ferrocyanide in the medium was 200 ppm and there was a slight change in its concentration during fermentation.

This was in agreement with the observation of Clark.[8] The slight decrease in the level of free ferrocyanide in the course of fermentation seems to be due to the accumulation of citric acid which results in its decomposition at low pH. Further increase in the concentration of ferrocyanide above 600 ppm or free ferrocyanide above 200 ppm resulted in lowering citric acid formation, sugar utilization and mycelial dry weight.

Filamentous and gelatinous growth in control cultures resulted in lowering the aeration of oxygen supply to the cultures thus adversely affecting citric acid production. The mould growth however, was modified to the form of separate, smooth and round pellets by the addition of ferrocyanide. This resulted in an increase in the aeration or oxygen supply to cultures which increased citric acid yield. The optimum pH of the molasses media was 6.0. At pH 3.0 the mould growth was inhibited when ferrocyanide was added to the medium at the time of inoculation. Martin[6] reported that toxicity of ferrocyanide is increased at low pH. Clarification of the media improved citric acid production, only in the molasses samples of Mardan and Charsadda; it may be due to the removal of some citric acid inhibitors during clarification.

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