http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 5(3): 321-324, 2002

Production of Calcium Gluconate by *Aspergillus niger* in Shake Flasks

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Abstract: The present study describes the production of calcium gluconate by locally isolated strains of *Aspergillus niger* in shake flasks using glucose salt, $CaCO_3$ medium. Thirty cultures of *Aspergillus niger* were isolated from different soil samples and were examined for acid production. Among all the cultures tested, *Aspergillus niger* IC-15 gave better production of calcium gluconate (85.50 g/l), 72 hours after spore inoculation. The cultural conditions optimized for maximum calcium gluconate production were, glucose concentration (150 g/l), pH (6.5) and $(NH_4)_2SO_4$ (2.5 g/l).

Key words: Calcium gluconate, *Aspergillus niger*, production, gluconic acid, glucose oxidase, production

Introduction

Calcium gluconate i.e., D-gluconic acid calcium salt ($C_{12}H_{22}CaO_{14}$) is one of the most important salts of calcium, which occurs as a white crystalline or granular powder, without taste or odor. It is widely used in food, textile, and leather, pharmaceutical, chemical and concrete industries and this has stimulated various scientists to undertake intensive investigation for increased production of the salt to meet its commercial demand (Buzzini *et al.*, 1993; Pedrosa *et al.*, 2000). It is stable in air and its solution is neutral to litmus paper. It is insoluble in alcohol and many other organic solvents (Traeger *et al.*, 1991).

Calcium gluconate decomposes by mineral acids and other acids, which are stronger than the gluconic acid. It is incompatible with soluble sulphates, carbonates, bicarbonates, citrates, tartrates, salicylates and benzoates. Calcium gluconate fills the need for a soluble; non-toxic well tolerant form of calcium. Calcium therapy is indicated in conditions such as parathyroid deficiency (tetany), general calcium deficiency (during pregnancy, growth, lactation, decreased dietary calcium intake, menopause, old age etc.) and when calcium is the limiting factor in increased clotting time of the blood. It can be used orally, intramuscularly and intravenously (Delgado and Remers, 1991; Ray and Banik, 1999).

Gluconic acid and its salts are commercially produced by three different methods; I- Chemical oxidation of glucose with a hypochlorite solution, ii- Electrolytic oxidation of glucose solution containing a measured amount of bromide (Amberkar *et al.*, 1965) and, iii- Fermentation process where specific micro-organisms are grown in medium containing glucose and other ingredients (Hill and Robinson, 1988; Shah and Kothari, 1993; Lee *et al.*, 1998). Oxidation of glucose to gluconic acid presents incomplete utilization of the sugar, this acid is known to be produced by several microorganisms. Gluconic acid was considered to be the product of incomplete oxidation of glucose to gluconic acid. However, later studies revealed that enzyme activity of some oxidases and dehydrogenases is responsible for oxidizing glucose to gluconic acid (Rose, 1961; Pons *et al.*, 2000).

The main object of the present work is to isolate and select a potent strain of *Aspergillus niger* from local habitats capable of producing large amounts of calcium gluconate.

Materials and Methods

Isolation of organism: Thirty isolates of *Aspergillus niger* were obtained from soils of different selected areas of Lahore by "Standard pour plate method". The mould cultures were screened by submerged fermentation method, using shake flasks each containing 25 ml of fermentation medium. All the experiments were performed in triplicates. The soil samples were taken from different areas of Lahore in polythene bags. This work is a part of M.Phil thesis (2001) at University of the Punjab, Lahore. The method of Johnson *et al.* (1959) was used for the isolation of *Aspergillus niger* strains. The young colonies of *Aspergillus niger* were picked up and grown on potato dextrose agar slants (BDH Germany) for culture maintenance. Conidia from a young colony of *Aspergillus niger* were then inoculated on solidified PDA slants.

Inoculum preparation: The spore suspension from an agar slant of 3-5 days old culture of *Aspergillus niger* was prepared by adding 10 ml sterile Monoxal O. T. (Dioctyl ester of sodium sulpho succinic acid). The conidial suspension was aseptically transferred to a sterile empty MacConkey bottle containing glass beads. The mixed suspension was shaken vigorously to break the clumps of conidia. The number of conidia per ml of inoculum was determined with the help of Thomas Counting Chamber.

Fermentation technique: Calcium gluconate fermentation was carried out by submerged fermentation in 250 ml cotton wool plugged Erlenmeyer flasks with 25 ml of fermentation medium containing (g/l); glucose 150, $(NH_4)_2SO_4$ 2.5, $MgSO_4.7H_2O$ 0.5, KH_2PO_4 1.0, $CaCO_3$ 32.0 having pH 6.0. The medium was inoculated by transferring 1.0 ml of conidial suspension (3×10^6 conidia/ml). The flasks were placed on a rotary shaker (model: GFL 544) for incubation at 30± 1°C with an agitation speed of 200 rpm for 72 hours. All the experiments were carried out in duplicate. The nitrogen sources and their concentration have marked influence on the metabolic system of the mould culture. Thus, the effect of adding various nitrogen sources on the production of mycelial dry weight, glucose consumption and calcium gluconate formation was investigated (Table 5). The nitrogen sources added were NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$ or peptone.

Analytical methods: The samples for analysis were taken out at the end of fermentation and the contents of flasks were filtered through Whatman No. 1. filter paper. Then the filtrate was centrifuged at 5000 rpm for 10 minutes. The supernatant liquid was used for estimation of glucose as well as for calcium gluconate.

Estimation of glucose: DNS (Dinitro salicylic acid) method was used for glucose estimation (Tasun *et al.*, 1970). The transmittance was measured at 530nm by spectrophotometer (Hitachi model: U-2000, Japan).

Estimation of calcium gluconate: The fermented broth was centrifuged and the supernatant liquid was used for analysis of calcium gluconate by the method of Pharmacopoeia (1990).

Mycelial dry weight: The mycelial dry weight was determined according to Chaturvedi *et al.* (1978).

Results

Screening of *Aspergillus niger* strain: The amount of calcium gluconate produced by different isolated mould cultures, ranged from 42.35 to 85.50 g/l. The mycelial dry weight was ranging from 6.50 to 15.50 g/l (Table 1). The *A. niger* IC-15 performed better as it gave the highest (85.50 g/l) production of Ca-Gluconate.

Rate of calcium gluconate production: The data in Table 2 shows the rate of Ca-gluconate production by *Aspergillus niger* IC-15. After 12 hours inoculation, the production of Ca-gluconate was

Table 1: Screening of *Aspergillus niger* for the production of Calcium gluconate in shake flask

Strain	Glucose (g/l)		Calcium gluconate produced (g/l)	% age yield of Calcium gluconate	Mycelial dry weight (g/l)
	Residual	Used			
IC-1	65.23	84.77	71.49	47.66	7.98
IC-2	70.25	79.75	66.48	44.32	8.58
IC-3	68.10	81.90	77.50	51.66	8.15
IC-4	69.30	80.70	75.80	50.30	8.45
IC-5	72.40	77.60	68.63	45.75	8.80
IC-6	63.50	86.50	60.70	40.76	10.00
IC-7	66.10	83.90	80.20	53.46	7.20
IC-8	74.25	75.75	70.10	46.73	7.10
IC-9	80.50	70.50	66.00	44.00	9.90
IC-10	86.50	63.50	55.25	36.83	11.20
IC-11	68.75	81.25	75.15	50.10	9.40
IC-12	98.00	52.00	45.25	30.16	8.75
IC-13	65.75	84.25	78.90	52.60	7.50
IC-14	95.20	54.80	45.50	30.33	10.60
IC-15	71.60	78.40	85.50	57.00	6.76
IC-16	80.00	70.00	63.80	42.53	8.25
IC-17	73.50	76.50	72.25	48.16	8.90
IC-18	81.50	68.50	63.20	42.13	9.75
IC-19	67.20	82.80	78.00	52.00	7.50
IC-20	100.25	49.85	42.35	28.23	14.75
IC-21	62.50	87.50	82.45	54.96	6.75
IC-22	66.00	84.00	80.12	53.41	6.85
IC-23	83.40	66.60	58.64	39.09	10.50
IC-24	71.50	78.50	73.50	49.00	8.00
IC-25	96.80	53.20	56.75	37.83	12.50
IC-26	69.40	80.60	83.10	55.40	6.50
IC-27	84.25	65.75	66.80	43.20	15.00
IC-28	100.15	49.85	48.20	32.13	15.50
IC-29	77.60	72.40	78.80	52.53	7.90
IC-30	82.50	67.50	65.50	43.66	10.50

Glucose added = 150 g/l pH = 6.0 Temperature = 30°C Incubation period = 72 hours Agitation = 200 rpm

Table 2: Rate of Calcium gluconate production by *Aspergillus niger* IC-15

Incubation period (hrs)	Glucose (g/l)		Calcium gluconate produced (g/l)	% age yield of Calcium gluconate	Mycelial dry weight (g/l)
	Residual	Used			
12	127.75	22.25	0	0	3.0
24	100.50	49.50	38.60	25.73	4.50
36	90.50	59.50	46.75	31.16	5.80
48	81.60	68.40	55.25	36.83	6.50
60	75.40	74.60	65.25	43.50	7.10
72	63.66	86.44	96.35	64.23	8.00
84	55.10	94.90	89.15	59.43	7.80
96	37.10	112.90	79.75	53.16	8.50
108	28.32	121.68	75.82	50.54	9.50
120	15.15	134.85	70.33	46.88	10.00

Glucose residual = Glucose added - Glucose used Glucose added = 150 g/l pH = 6.0 Temperature = 30°C

Table 3: Effect of pH on the production of Calcium gluconate by *Aspergillus niger* IC-15

Incubation period (hrs)	Glucose (g/l)		Calcium Gluconate produced (g/l)	% age yield of Calcium Gluconate	Mycelial dry weight (g/l)
	Residual	Used			
4.0	105.60	44.40	29.50	19.66	3.88
4.5	94.25	55.75	49.75	33.16	4.70
5.0	70.10	79.90	69.35	46.23	5.75
5.5	66.45	83.55	86.76	57.84	7.98
6.0	50.25	993.75	98.36	65.57	8.40
6.5	40.20	109.80	112.77	75.18	8.96
7.0	62.70	87.30	86.37	57.58	8.75
7.5	69.25	80.75	79.36	52.90	9.50
8.0	60.30	89.70	72.75	48.50	9.65

Glucose residual = Glucose added – Glucose used Glucose added = 150 g/l Fermentation period = 72 hours Rotation rate = 200 rpm

Table 4: Effect of glucose concentrations on the production of Calcium gluconate by *Aspergillus niger* IC-15

Incubation period (hrs)	Glucose (g/l)		Calcium gluconate produced (g/l)	% age yield of Calcium gluconate	Mycelial dry weight (g/l)
	Residual	Used			
10.0	40.00	60.00	49.00	49.00	6.20
12.5	41.65	83.35	80.00	64.00	7.10
15.0	39.50	110.50	112.35	74.90	8.70
17.5	66.25	108.75	109.00	62.28	9.80
20.0	87.50	112.50	115.00	57.50	10.30
22.5	113.15	111.85	110.33	49.03	12.75
25.0	140.25	109.75	112.20	44.88	13.25
27.5	170.45	104.55	107.33	39.02	13.80
30.0	210.15	89.85	85.44	28.48	14.20

Glucose residual = Glucose added – Glucose used Glucose added = 150 g/l Fermentation period = 72 hours Rotation rate = 200 rpm

Table 5: Effect of nitrogen sources on the production of Calcium gluconate by *Aspergillus niger* IC-15

Nitrogen sources	Glucose (g/l)		Calcium gluconate produced (g/l)	% age yield of Calcium gluconate	Mycelial dry weight (g/l)
	Residual	Used			
NH_4NO_3	45.25	104.75	103.50	69.00	7.31
$(NH_4)_2SO_4$	40.67	109.33	120.10	80.66	8.23
NH_4Cl	63.67	86.33	82.18	54.78	10.78
Peptone	51.75	98.25	96.77	64.51	10.00
$NaNO_3$	77.00	73.00	61.75	41.16	12.99

Fermentation period = 72 hours Nitrogen source added = 2.5 g/l

Table 6: Effect of different concentration of ammonium sulphate on the production of Calcium gluconate by *Aspergillus niger* IC-15

Amount of Ammonium sulphate	Glucose (g/l)	Calcium gluconate produced (g/l)	% age yield of Calcium gluconate	Mycelial dry weight (g/l)
1.0	91.55	88.25	58.83	7.35
1.5	105.25	107.00	71.33	8.10
2.0	120.23	125.33	83.55	8.25
2.5	125.88	110.20	73.46	8.90
3.0	111.66	89.22	59.48	9.33
3.5	92.75	69.88	46.58	9.27
4.0	97.65	62.36	41.57	11.98

pH = 6.5 Glucose added = 150 g/l Fermentation period = 72 hours Temperature = 30°C

negligible. However its production was increased 24 hours after spore incubation & at 48 hours, Ca-gluconate production was about 55.25 g/l. The production of Ca-gluconate was 65.25 g/l at 60 hours incubation. The production reached its maximum i.e., 96.35 g/l at 72 hours of incubation. Further increase in the incubation period, resulted in lowering the yield of Ca-gluconate. The mycelial weight and glucose consumption was also increased during fermentation. The amount of mycelial dry weight was 8.00 g/l, 72 hours after spore inoculation.

Effect of pH: Table 3 shows the effect of pH (4.0 – 7.5) on Ca. gluconate production. The pH of fermentation medium was adjusted with 1N HCl or NaOH solution. The production of Ca-gluconate was minimum at pH 4.0 (i.e., 29.50 g/l) and mycelial dry weight was also poor at this pH. Raising the pH from 4.0 to 6.0 increased the rate of calcium gluconate production. It was maximum (i.e., 112.77 g/l) at pH 6.5. Thus it was used in further research. Rate of Ca- gluconate production was again decreased above this pH i.e., about 72.75 g/l at pH 8.0.

Effect of glucose concentration: The concentration of carbon sources plays an important role on the conversion of glucose into gluconic acid and its calcium salt. The data in Table 4 shows the effect of different glucose concentration (10-30 % w/v) on the consumption of glucose, mycelial dry weight and calcium gluconate formation. In all of these levels of sugar, the maximum conversion into gluconic acid was found when the glucose concentration was 15 % w/v i.e., 11.35 g/l (74.90 %). The production of calcium gluconate however was reduced with the increase in concentration of sugar. The mycelial growth was increased at higher concentration of glucose added.

Effect of nitrogen sources: The glucose consumption, hence its conversion into calcium gluconate was maximum in the presence of ammonium sulphate i.e. 109.33 g/l and 120.10 g/l respectively. Thus ammonium sulphate was selected for further experiments. The mycelial dry weight produced by various nitrogen sources was ranged from 7.31 to 12.99 g/l in the presence of ammonium sulphate, it was 8.23g/l.

Effect of different concentrations of ammonium sulphate: Effect of different concentrations of ammonium sulphate (1.0-4.0 g/l) on the growth of *Aspergillus niger* IC-15 and calcium gluconate formation was investigated (Table 6). The optimum level of nitrogen source was 2.0 g/l. The glucose consumption, calcium gluconate formation and mycelial dry weight were 120.23, 125.33, and 8.25g/l respectively. At a concentration of 1.0 and 1.5g/l of ammonium sulphate the yields of calcium gluconate were 88.25 and 107.00 g/l respectively. However the yield also decreased with the increase in level of ammonium sulphate. The mycelial dry weight ranged from 1.98 to 7.35 g/l and its growth was directly proportional to the amount of ammonium sulphate.

Discussion

The production of calcium gluconate by *Aspergillus niger* is a worth praising achievement in the field of Fermentation Technology. The optimization of cultural conditions such as pH, temperature, incubation period & selection of the suitable substrate is very essential for maximum production of enzyme glucose oxidase and the bioconversion of glucose into calcium gluconate or gluconic acid (Prescott & Dunn's, 1987; Yoshie 1999). The present studies describe the production of calcium gluconate by *Aspergillus niger*. Of all the cultures examined, *Aspergillus niger* IC-15 was selected as it gave better results of calcium gluconate. The selected culture consumed 86.44 g glucose, 72 h after conidial inoculation and the amount of calcium gluconate produced was about 96.35 g/l. The mycelial dry weight was 8.00 g/l. The initial concentration of the glucose in the basal medium was kept as 150 g/l.

Optimum fermentation period is one of the most important factors in calcium gluconate fermentation (Pons *et al.*, 2000). The time course fermentation studies during fermentation showed that the calcium gluconate production was maximum at 72 hrs, after conidial inoculation. Further incubation did not increase the production of calcium gluconate which might be due to the over growth of mycelium. The results also revealed that actual biosynthesis of the product was started 24 h after inoculation as there was no production prior to this period. Buzzini *et al.* (1993) have reported maximum yield of calcium gluconate at 72 h of time. However, according to Yasin *et al.* (1975) the maximum amount of calcium gluconate was produced by *Aspergillus niger* strain was 96 h after spore inoculation. Hence, the present finding is more encouraging as compared to Yasin *et al.* (1975) because reduction in the time period reduced the cost of calcium gluconate production.

The maintenance of a favourable pH is very essential for the successful fermentation of calcium gluconate (Milikovic & Vukojevic, 1989; Lee *et al.*, 1998). The influence of pH of fermentation medium on the growth of the mould culture and hence the concentration of gluconic acid was also investigated. At an initial pH of 5.0 and less than 5.0, both mycelial growth and calcium gluconate production was poor while at initial pH of 6.5, the consumption of glucose as well as calcium gluconate production was improved significantly. It may be due the fact that at pH 6.5, the mycelium contained maximum glucose oxidase. However, further increase in the initial pH reduced the rate of

bioconversion of glucose into calcium gluconate. These results are actually in accord with the work of Takao & Sasaki (1964). At a lower pH, glucose oxidase was not formed yet its formation started at pH value greater than 5.0. Therefore, the effect of initial pH on calcium gluconate production might reflect the effect on glucose oxidase activity. In a similar study, Ray and Banik (1999) found maximum amount of calcium gluconate by mutant *Aspergillus niger* at a pH 6.5. However, the study is not in good agreement with Kundu and Das (1984), who got maximum yield of calcium gluconate at initial pH 5.5. This may be due to the type of strain and its physiological conditions. As the pH increased up to 7.0 or 8.0, the production of calcium gluconate was remarkably decreased.

The initial glucose concentration has been found to determine the amount of calcium gluconate by *Aspergillus niger*. Effect of glucose concentration on calcium gluconate production and mycelial dry weight was investigated. When glucose concentration in the medium was 15% (w/v) the yield of calcium gluconate was maximum. However, further increase in the glucose concentration resulted in lowering both the glucose utilization and calcium gluconate formation. Increase in calcium gluconate yield at 150 g/l of glucose might be due to the fact that at this concentration of glucose, oxidase activity in *Aspergillus niger* was optimum which resulted in the high yield of calcium gluconate. The decrease in the production may be due to catabolic repression of *Aspergillus niger* (Doneva *et al.,* 1999). Qadeer *et al.* (1975) found that 10-15 % of glucose is more suitable and economical, for calcium gluconate production and when glucose concentration was increased up to 30%, the yield of calcium gluconate was markedly decreased. Moreover, at a higher concentration of glucose (20 - 30 %), the occurrence of foam was noted which disturbed the proper mycelial distribution. Mycelium accumulated on the foam layer decreased the contact surface of mycelium with fermentation medium, and this resulted in slower bioconversion of glucose to calcium gluconate. A glucose concentration of 15% was found optimum for calcium gluconate production. Ray and Banik (1994) have also observed higher yield of calcium gluconate when the concentration of glucose was 150 g/l than any other concentration of glucose. They found 100% of yield from this substrate concentration. Our observation is similar to the above-mentioned workers.

Nitrogen constituent has a profound effect on the yield of gluconic acid because the type of nitrogen source and its concentration affect the performance of the fungus considerably. Effect of different nitrogen sources on both the glucose consumption as well as calcium gluconate production was examined. The most suitable nitrogen source for the production of calcium gluconate by *Aspergillus niger* IC-15 was ammonium sulphate. However, sodium nitrate resulted in minimum yield of calcium gluconate production. The other nitrogen sources like ammonium nitrate, ammonium chloride, sodium nitrate and peptone were favourable for mycelial growth of *Aspergillus niger* but not suitable for calcium gluconate production. In a similar study, Elnaghy and Megalla (1975) have reported that the peptone was the best source for the production of calcium gluconate. But in present series of experiments, ammonium sulphate was found to be the best source of nitrogen for the production of calcium gluconate so its various concentrations (1.0-40 g/l) were further studied. The optimum level of $(NH_4)_2SO_4$ was 2.0 g/l. Further increase in its concentration did not enhance the production of calcium gluconate. However, at higher concentrations of nitrogen, the growth rate of the fungus was increased but the amount of glucose provided in medium was consumed only for the growth of *Aspergillus niger* and not for the production of Ca-gluconate.

Thus only 2.0 g/l of ammonium sulphate was optimum and used in further studies. From the studies it is evident that the biosynthesis of calcium gluconate is strongly dependent on the selection of strain as well as the cultural conditions employed.

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