PRODUCTION OF CALCIUM GLUCONATE BY *ASPERGILLUS NIGER GCU-1*

K. Shahzadi, S. Naz, A. Tariq, S. Ilyas, M. A Qadeer* and I. U. Haq*

Department of Biotechnology &Microbiology, Lahore College for Women University, Lahore, Pakistan
*Institute of Industrial Biotechnology, GCU, Lahore, Pakistan
Corresponding Author: E-mail: kiran.qamar@yahoo.com

ABSTRACT: Calcium gluconate was produced in shake flasks and scale up investigations in 7.5L New Brunswick Scientific glass fermentor with a working volume of 5Lby locally isolated strain of *Aspergillusniger GCU-1* were also carried out. The glucose salt-$CaCO_3$ medium was employed for fermentation. The culture conditions using 25ml cotton wool plugged conical flasks were period of incubation (0-96hrs),the concentration of glucose (5.0-125g/l) and the concentration of $(NH_4)_2HPO_4$ (0.00-4.0g/l). The period of incubation was optimized at 48 hrs after inculation. The glucose concentration and the concentration of $(NH_4)_2HPO_4$were also optimized to 125g/l and 0.4g/l, respectively. Scale up studies in the fermentor reached maximum 48 hrs after the addition of 24hrs old vegetative inoculum. The reuse of fungal mycelium for calcium gluconate fermentation was also investigated. The results were encouraging up to third generation as its further use resulted in the reduced consumption of glucose. A completely randomized design was used for the parameters with three replicates. The data was analyzed by COSTAT V.63: statistical software (Cohort software, Berkely, California) and Duncan' New Multiple Range.As theoretical yield of calcium gluconate is more than 100% so the development of this technology would be highly feasible.

Key words: Calcium gluconate, *Aspergillusniger*, Production, Shake flask, Fermentor.

INTRODUCTION

Calcium gluconatei. e, D- gluconic acid calcium salt ($C_{12}H_{22}CaO_6$) is considered to be the most important salts of calcium which occurs in two morphological forms, one is white crystalline substance or granular and the other is powder which is tasteless and odorless. This salt is widely used in food, textile, leather, pharmaceutical industry and concrete production and this has stimulated various scientists towards intensive investigation for increased production of the salt to meet its commercial demand (Buzzini*et al*., 1993; Syed *et al*., 1996; Pedrosa and Serrano, 2000, Tripathi *et al*., 2001). It shows stability in air and shows neutral reaction towards litmus paper. It shows insolubility towards organic solvents, e.g. alcohol (Trager and Qazi, 1991).

Gluconic acid is much stronger than mineral acids and other acids which can decompose calcium gluconate. It shows no compatibility as compared to soluble sulphate, carbonates, bicarbonates, citrates, tartarates, salicylates and benzoates. Calcium gluconate fulfills the requirement for a soluble, non toxic, well tolerated form of calcium (Taha*et al.*, 1960). In conditions such as parathyroid deficiency (tetany), general calcium deficiency (during pregnancy, growth, lacation, decreases dietary calcium intake, menopause, old age etc.) calcium therapy is indicated. When calcium is the limiting factor in increased clotting time of the blood it can be used orally, intramuscularly and intravenously (Ray and Banik, 1994).

Commercially, gluconic acid salts are produced by three different methods; 1- Electrolytic oxidation of glucose containing a measured amount of bromide (Ambekar*et al*., 1965) 2- Chemical oxidation of glucose with a hypo-chlorite solution (Kundu and Das, 1984) 3- Fermentation process where specific microorganism are grown in medium containing other ingredients (Hill and Robinson, 1988). The incomplete oxidation of glucose results in the production of gluconic acid which is being done by using several types of microorganisms. The strains of *Aspergillusniger* have higher capability of producing calcium gluconate than other strains of microorganisms(Kundu and Das, 1982; Kaczmarowicz and Walisch, 1983, Baajaree and Kannika, 1993).The aim of the work was to produce calcium gluconate by using a strain of *Aspergillusniger* in both shake flasks and stirred fermentor using glucose salt $CaCO_3$ medium.

MATERIALS AND METHODS

Organism: The locally isolated strain of *Aspergillusniger GCU- 1* was taken from the culture collection of "Institute of Industrial Biotechnology GCU" Lahore. The culture was maintained on the potato dextrose agar medium.

INOCULUM PREPARATION:

a) Spore Inoculum: The spore suspension from a 100 ml conical flask containing 3-5 days old germinated spores of *Aspergillusniger* was prepared by adding 10 ml

sterilized Monoxal O.T (Dioctyl ester of sodium sulpho succinic acid).The conidia were scratched with inoculating needle and the flasks were rotated gently to break the clumps of conidia (Yasin*et al*.1975). The number of conidia per ml of inoculum was determined with Haemacyto meter (Tiefe depth profounder 0.0025 mm^2 area).

b) Vegetative Inoculum: For fermentor studies 10% vegetative inoculum was prepared by inoculating the flasks containing germination medium g/l Glucose 60.0, Corn steep liquor 30.0, $MgSO_4.7H_2O$ 0.20, KH_2PO_4 0.24, Urea 0.102, $(NH_4)_2 PO_4$ 0.407 and yeast extract 5.0.

FERMENTAION TECHNIQUE: The production of calcium gluconate was carried out in both shake flasks and glass fermentor. The fermentation medium contains/l Glucose 120l, $(NH_4)_2PO_4$ 0.39, KH_2PO_4 0.19, $MgSO_4.7H_2O$ 0.16, Corn steep liquor 3.00 and $CaCO_3$ 40.00.All the ingredients except $CaCO_3$ were dissolved in distilled water and autoclaved at 121^0C for 15 min. $CaCo_3$ was sterilized separately and mixed aseptically with the rest of medium at time of inoculation. The pH of the medium was adjusted to 7.00 by 1N NaOH or 1N HCL.

a) **Shake flask:** Calcium gluconate fermentation was carried out by submerged fermentation in 250 ml cotton plugged Erlenmeyer flasks containing 25 ml of fermentation medium. The medium was inoculated by transferring 1.0 ml of conidial suspension (2.5×10^7 conidia/ml).The flasks were placed at rotary shaker for incubation at 30 ± 1^0C with an agitation speed rate of 200 rpm for 48 hours. All the experiments were carried out in duplicate.

b) **Stirred fermentor:** Calcium gluconate fermentation was also carried out in 7.5L glass fermentor with a working volume of 5L. The fermentor containing basal medium was sterilized in an autoclave at 121^0C for 10 minutes and cooled to 30^0C. The medium was aseptically inoculated by 10% vegetative inoculum developed in shake flasks and also added separately sterilized $CaCo_3$ suspension. To control foaming sterilized silicon was use.

Analysis: The samples for analysis were taken by filtering the fermentation medium through Whatman No.1 filter paper. Then the filtrate was centrifuged and the supernatant liquid was used for estimation of glucose as well as calcium gluconate.

Glucose estimation: The estimation of glucose was carried out by DNS (Dinitro salicylic acid) method (Tasunand Ghen 1970). The transmittance was measured by spectrometer at 575nm.

Estimation of calcium gluconate: A known volume of supernatant was taken in the test tubes. Then added an equal volume of methanol and Ethyl- Ether in 1:1 ratio, and placed in cool cabinet at 4^0C for overnight. After 24 hours the contents of calcium gluconate were filtered through weighed Whatmann filter paper No.1 and dried at 100^0C for 24 hours. The weight of calcium gluconate was calculated by subtracting the weight of filter paper from the total dry weight.

Mycelial dry weight: The determination of mycelial dry weight was according to Chaturvedi*et al*., 1978).

RESULTS AND DISCUSSION

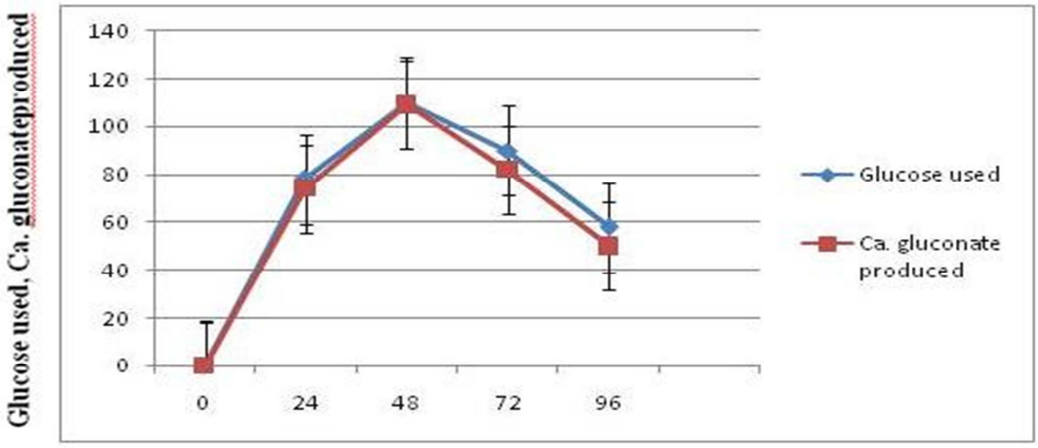
The present study is concerned with the production of calcium gluconate by *Aspergillusniger GCU-1*. Glucose salt medium (25ml) containing $CaCO_3$ was used as the basal fermentation medium for the production of calcium gluconate. The conidial suspension prepared in Monoxal O.T. was used as an inoculum for fermentation studies.

The time course fermentation studies during fermentation showed that the calcium gluconate production was maximum 48 hours after spore inoculation, (Fig-1). Further incubation did not enhance the production of calcium gluconate which might be due to the over growth of mycelium. The result also revealed that actual biosynthesis of the product was started 48h after Inoculation as there was no production prior to this period. Buzzini *et al*., (1993) however have reported maximum yield of calcium gluconate 72h after inoculation and according to Yasin*et al*.(1975) the maximum amount of calcium gluconate was produced by *Aspergilllusniger* strain 96h after inoculation. Hence the present finding is more encouraging as compared to because reduction in the time period reduced the energy consumption during calcium gluconate production.

The concentration of carbon plays an important role in the conversion of glucose into gluconic acid and its calcium salt. Effect of glucose concentration (5.0%-12.5% w/v) on calcium gluconate production was investigated (Table-1). When glucose concentration in the fermentation medium was (12.5% 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 %age yield of calcium gluconate at 125 g/l glucose concentration might be due to fact that at this concentration, glucose oxidase activity in *Aspergillusniger* was maximum which resulted in the high yield of calcium gluconate. The decrease in the production may be due to catabolic repression of *Aspergillusniger* (Sukuri and Lee, 1989; Donev*aet al*., 1999).

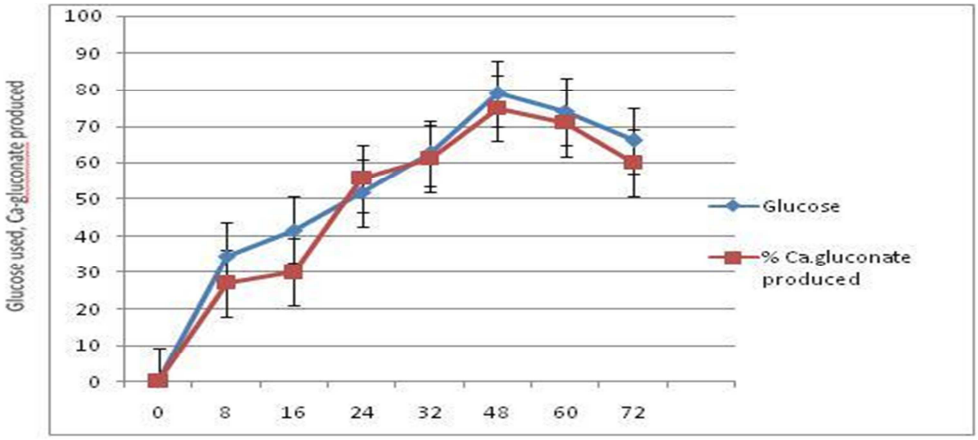
Nitrogen constituent has a profound effect on the yield of gluconic acid because the type of nitrogen source and its concentration affect the performance of the fungus considerably (Kapoor *et al*., 1987). Effect of

different nitrogen sources on both the glucose consumption as well as calcium gluconate production was examined.



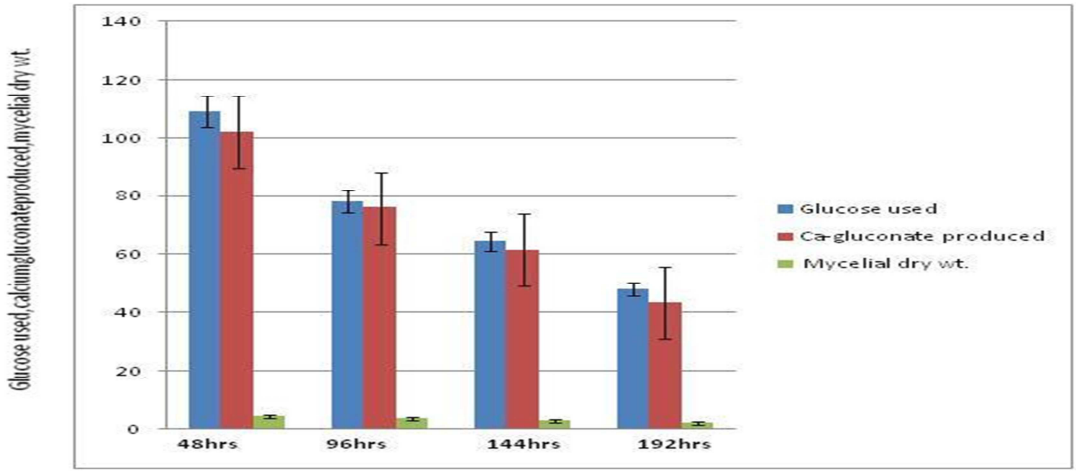
Glucose residual= Glucose added-Glucose used Glucose added=125g/l Fermentation period=48 hours Agitation rate= 200rpm.

Fig – 1.



Glucose residual= Glucose added-Glucose used Glucose added=125g/l Fermentation period=48 hours Agitation rate=200rpm

Fig-2. Scale up production of calcium gluconate by *Aspergillusniger GCU-1* in the stirred fermentor



Glucose residual= Glucose added-Glucose used Glucose added=125g/l Fermentation period=48 hours agitation rate=200rpm in stirred fermentor

Fig -3. Effect of reuse of fungal mycelium on the production of calcium gluconate by *Aspergillusniger GCU*-1

Table-1. Effect of glucose concentration on the production of Ca. gluconate by *Aspergillusniger GCU-1* in shake flasks.

Concentration of glucose (%)	Glucose		Calcium gluconate produced(g)	%age yield of calcium gluconate	Mycelial dry weight (g)
	Residual	Used			
5.0	0.00	50.0	38.082^{d} ±0.257	31.056	2.295^{f}± 0.109
7.5	0.00	75.0	46.880^{c}±0.298	37.344	3.560^{de}±0.212
10.0	23.00	77.0	55.345^{b}±0.187	44.276	5.590^{ab}±0.196
12.5	26.00	99.0	84.568^{a}±0.214	75.654	6.340^{a}±0.126
15.0	50.00	100.00	75.876^{ab}±0.296	35.108	5.820^{ab}±0.328
LSD	-	-	1.0298	--	0.9322

Each value is mean of three replicates with standard error (mean ± S.E). Different letters in the same column indicates significant differences at p=0.05 according to Duncan's new multiple range test.
Glucose residual= Glucose added-Glucose used
Glucose added=125g/l Fermentation period=48 hours Agitation rate= 200 rpm
LSD=Least significant difference Significance level≤0.05

Table 2: Effect of $(NH_4)_2HPO_4$ concentration on the production of Ca. gluconate by *Aspergillusniger GCU-1*

Concn. of $(NH_4)_2HPO_4$ (g)	Glucose		Ca. gluconate produced (g)	%age of yield Ca. gluconate	Mycelial dry wt. (g)
	Residual (g)	Used (g)			
0.00	37.0	88.0	70.453^{d}±0.191	56.356	2.567^{b}±0.149
0.20	29.5	95.5	86.972^{b}±0.292	66.359	2.987^{ab}±0.281
0.4	17.0	108.0	106.560^{a}±0.201	85.248	4.144^{a}±0.052
0.6	34.0	91.0	83.341^{c}±0.085	68.278	3.463^{ab}±0.131
0.8	48.0	77.0	68.459^{e}±0.132	54.767	3.545^{ab}±0.155
LSD	-	-	1.0886	-	1.099

Each value is mean of three replicates with standard error (mean ± S.E). Different letters in the same column indicates significant differences at p=0.05 according to Duncan's new multiple range test.
Glucose residual= Glucose added-Glucose used
Glucose added=125g/l Fermentation period=48 hours Agitation rate= 200 rpm
LSD=Least significant difference Significance level≤0.05

The most suitable nitrogen source for the production of calcium gluconate by *Aspergillusniger GCU-1* was di-ammonium hydrogen phosphate (Arati and Nandi, 1969). The data of (Table-2) shows the effect of its concentration (0.4-0.8 g/l). The optimum level of di ammonium hydrogen phosphate was 0.4g/l. further increase in its concentration did not enhance the production of calcium gluconate. However, at higher concentration of nitrogen, the mycelial formation was increased but the amount of glucose provided into the medium was consumed only for the growth of *Aspergillusniger GCU-1* and not for the production of calcium gluconate. Thus 0.4 g/l of di-ammonium hydrogen phosphate was optimized and used for the fermentation.

The reuse of fungus mycelium as a source of glucose oxidase was also investigated for the conversion of glucose into gluconic acid. It may reduce the incubation period as well asincrease the production of calcium gluconate. Large number of workers has described the conditions for re-use of mycelium in gluconate fermentation (Niazi*et al*., 1969; Sulema*et al*., 1998; Pedrosa*et al*., 2000). Fig 3 depicts the production of calcium gluconate by repeatedly using the mycelium of *Aspergillusniger GCU 1* obtained by asepticcentrifugation of fermented broth. The mould mycelium after each fermentation was separated from fermented broth under aseptic conditions using sterile centrifuge tubes. The mycelium thus obtained from previous batch was transferred to fresh sterile medium contained in shake flasks. In the first batch, the amount of calcium gluconate produced was 102.567 g/l and glucose consumption was 113.45 g/l. Further use of fungal mycelium however resulted in lowering the consumption of both glucose and production of calcium gluconate as 71.00 and 68.400 g/l, respectively. The reuse of fungal mycelium for the next batch greatly reduces both glucose consumption and calcium gluconate production. This experiment is applicable in industries where time and labor cost is important factors for the manufacturing of calcium gluconate or other products. Ray and Banik, (1994) reported the reuse of fungus mycelium of calcium gluconate production.

After optimizing the cultural condition in the shake flasks, the calcium gluconate fermentation was also carried out in 7.5L glass ferment or with a working volume of 5L. The sterilized medium was aseptically inoculated by 10% vegetative inoculums developed in

shake flasks and also added separately sterilized $CaCO_3$ suspension. The sterilized silicone was used to control foaming. From the results of fig 2 it is evident that the maximum yield of calcium gluconate was 48h after inoculation. Further increase in incubation time period resulted in lowering the yield o0f calcium gluconate.

From the studies it is evident that the biosynthesis of calcium gluconate is strongly dependent on the selection of strain as well as cultural condition employed.

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