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Optimisation of Glucose Fermentation for the Production of Calcium Gluconate in Terms of an Industrial Perspective

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Abstract:- An attempt has been made to convert glucose into calcium gluconate with the help of *Aspergillus niger* through fermentation. The present study focused to optimise fermentation parameters like glucose concentration, dose of inoculums, age of inoculums, pH, agitation, aeration and temperature, for developing an efficient, cost-effective, eco-friendly protocol for the production of calcium gluconate, also examine the reproducibility of optimised protocol and scale-up study to decide its techno-commercial viability.

The glucose fermentation protocol of various parameters optimized and subsequently an optimized protocol for the conversion of glucose into calcium gluconate was devised. It comprised (a) Optimal concentration of glucose 15%, (b) Optimal dose of inoculum 10%, (c) Optimal age of inoculum 24 hour, (d) Optimal pH 6 $\pm$0.2, (e) Optimal rate of agitation at 200 rpm, (f) Optimal rate of aeration 1.0 vvm, and (g) Optimal temperature 30$\pm$1^{0}C. By using this optimised protocol its scale up study showed that techno-commercial viability was feasible and it can be commercialized without any problems with desire quality product as required by pharmaceutical, food, feed, poultry, and aquaculture industries due to its bio-availability. The currently preferred technology for the production of gluconic acid and its derivatives is with the aid of *Aspergillus niger* due to least pollution, less energy consumption, and higher productivity.

Keywords:- *Calcium Gluconate, Gluconic Acid, Glucose, Fermentation, Aspergillus Niger.*

I. INTRODUCTION

Glucose typically acts as a carbon source for the microbial production of gluconic acid. However, hydrolysis of various raw materials such as agro-industrial waste has also served as a substrate [1]. Glucose is the main product of starch industry and has very high potential for value addition as a source of many salts of gluconic acid like calcium, magnesium, zinc gluconates etc. [2]. Glucose is readily and economically available in bulk, which may be processed using *Aspergillus niger* into gluconic acid. and then neutralisation with calcium carbonate to form calcium gluconate [3].

The products of *Aspergillus niger* are enzymes, organic acids, antibiotics and mycotoxins [4]. It is a prodigious exporter of species of homologous proteins and is able to produce certain enzymes in quantities of kg/m^3 under right conditions. It is also generally recognized as safe (GRAS) and has a long tradition of use in the fermentation industry [5]. Gluconic acid derived from glucose by a simple dehydrogenation reaction [6]. It is multi-functional organic acid used as a bulk chemical in the food; feed, pharmaceutical, textile, metallurgical, detergent and construction industries [7, 8, and 9]. Glucose oxidase (EC 1.1.3.4., D-glucose: oxygenoxidoreductase) catalyses the oxidation of D-glucose to glucono lactone ($C_6H_{10}O_6$) and hydrogen peroxidase using molecular oxygen as the electron acceptor. The worldwide production of gluconic acid is nearly about 87,000 tonnes /year and costs about 1.2.-8.50/kg of gluconic acid [10].

Calcium gluconate contains 9.3% calcium; its molecular formula is C12H22CaO14.H2O and has a molecular weight of 448.38[11]. Calcium gluconate commercially used to prevent and treat calcium deficiency, mainly in the pharmaceutical, food, feed and aqua industries [2].

A substantial amount of glucose is produced by the starch industry, but glucose is used as a carbon source in many fermentation industries [12]. In addition to these applications, it does not have many value-added uses attractive to the starch industry [13].

In view of its negligible alternate, the value addition, the easy and economical availability and limited literature on glucose bio- conversion in to calcium gluconate production. Towards these objectives, a number of experiments have been carried out to optimise the production parameters like pH, Temperature, inoculum doses, age of the inoculum, concentration of glucose, aeration, agitation and positive pressure for efficient, cost-effective, eco-friendly protocol. The reproducibility of the optimised protocol was explored using cost-effective media and scale up the reproducible protocol to determine its technical-commercial viability and the problems encountered during scale up.

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II. MATERIALS AND METHODS

Microbial culture, Chemicals and reagents

Aspergillus niger culture was provided by M/S KCP Sugars and Industries Corporation Limited, Vuyyuru, Andhra Pradesh. It was screened as the most efficient strain among the efficient species, for the fermentation of glucose to gluconic acid. Laboratory grade chemicals and reagents were used for spore formation.

Preparation of media for slant / spore formation

The medium was prepared as per the composition in Table 1 for *A. niger* slants.

Table 1: Composition of slant media

S. No.	Ingredient	grm/ liter
1	Agar-agar	20.00
2	Sodium chloride (NaCl)	10.00
3	Molasses	7.50
4	Glycerin	7.50
5	Yeast extract	5.00
6	Calcium sulphate ($CaSO_4$)	0.25
7	Trace elements(g/liter)	1.00 ml each
a)	KH_2PO_4	6.00
b)	$MgSO_4$	5.00
c)	Fe (NH_4) SO_4	1.60
d)	$CuSO_4$	1.00

Note: Molasses (1:2 dilution) were pre-sterilized for 30 min, at 121^{0}C at 15lbs pressure. The above medium with pH 5.5 to 5.8 was sterilized at 121^{0}C for 30 min at 15 lbs pressure.

Preparation of media for spore germination

It was prepared by using commercial grade chemicals as summarized in **Table 2**. The spore germination medium having pH 5.5 to 5.8 was sterilized at 121˚C for 15 minutes at 15 lbs pressure.

Table 2: Composition of media for spore germination

S. No.	Ingredient	grm /liter
1	Glucose	50.0
2	$MgSO_4$	2.5
3	DAP	2.0
4	KH_2PO_4	1.0

Preparation of media for the commercial production of calcium gluconate

The commercial production medium was prepared commercial grade chemicals as summarised in Table 3**.** The commercial production medium having pH 5.5 to 5.8 was sterilized at 121˚C for 15 minutes at 15 lbs pressure and calcium carbonate was sterilized separately.

Table 3: Composition of media for the production of calcium gluconate

S. No.	Ingredient	grm/liter
1	Glucose	150.00
2	DAP*	0.90
3	KH_2PO_4	0.20
4	$MgSO_4$	0.15
5	Urea	0.11
6	Calcium carbonate	55.00

*DAP = Di-ammonium Phosphate

Preparation of slants for spore formation.

The slant medium was prepared as per the composition given in Table 1 and poured into test tubes. These tubes were plugged with non-adsorbent cotton and sterilized at 121^{0}C for 15 minutes at 15lbs pressure. After sterilization, these tubes were kept in slanting position to make the slants. Thereafter, these slants were kept into incubator for 24 hours at 30˚C to confirm sterility and then slants were inoculated with *Aspergillus niger* spores under aseptic condition using LAF. After inoculation, these slants were incubated at 30˚C. *Aspergillus niger* spores formation completed within 36 to 96 hours.

Harvesting of *Aspergillus niger* spores

The spores of *A.niger* from the slants were harvested in 7.5ml pre-sterilized 0.1% Tween-80 solution. The harvested suspension was thoroughly mixed and spore concentration was determined using haemocytometer under microscope, as per classical procedure. The suspension of spores in the inoculum was maintained at 10^6 to 10^8 spores / ml. It was inoculated into spore germination medium as mentioned in Table 2, and put on orbital shaking incubator at 30^{0}C with 200 rpm for better agitation / aeration and monitored at regular interval.

Inoculum preparation of *Aspergillus niger* from spores

For this purpose, spore germination medium prepared as per Table 2 was sterilized at 121˚C for 15 minutes, at 15lbs pressure, the sterility was confirmed after 24 hours and inoculated with 10 ml of spore suspension concentration 10^6 to 10^8 spores/ml for 24 h, at 29±1^{0}C, 200 rpm in orbital shaking incubator.

Typical experimental set up

Calcium gluconate production medium (PM) was prepared as per Table 3, sterilized at 121˚C for 15 minutes, at 15lbs pressure and incubated for 24 hours at 30˚C in orbital shaking incubator for sterility confirmation. Thereafter, inoculated with 100 ml of 24 h old inoculum per 1000 ml of PM having spore concentration 10^6 to 10^8 spores/ml and incubated in orbital shaking incubator with 200rpm at 30^{0}C for 48 h to examine the kinetics of calcium gluconate formation. The parameters indicated in Table 4 were monitored at regular interval.

Table 4: Fermentation monitoring data sheet

Duration (h)	pH	Bio-mass		$CaCO_3$ (g)	RS (%)	CG (%)
		Wet (g%)	Dry (g%)			
00	5.50	4.40	0.80	Nil	15	0
8.0	3.50	4.60	0.82	9.0	14.0	2.1
24.0	5.51	2.80	0.80	13.0	1.90	6.72
48.0	5.66	2.80	0.80	Nil	1.80	6.72

RS = Reducing sugar, CG = Calcium gluconate

From **Table 4**, it is clear that within 8 h, pH of the production medium came down from 5.50 to 3.50,

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indicating that the formation of gluconic acid was underway. To neutralize it, pre-sterilized slurry of calcium carbonate was added to the broth to carry forward fermentation to optimum level. The fermentation was carried out for 48 h. It was considered desirable to optimize the calcium gluconate production parameters after the formation of calcium gluconate was established.

III. RESULTS AND DISCUSSION

In these studies, only one parameter was varied at a time, keeping other parameters unaltered. When a parameter was showing maximum growth promotion / gluconate formation at a particular point, that parameter was regarded as an optimum. Thus, different parameters likely to affect the production were sequentially optimized to evolve an optimum protocol. Towards this purpose, the following parameters were taken in consideration for optimization: 1. Dose of inoculum, 2. Age of inoculum, 3. pH, 4. Rate of agitation, 5. Rate of aeration, 6. Temperature, and 7. Concentration of glucose.

Dose of inoculum

The dose of inoculum for inoculation into the production media was at the rate of 5, 10 and 15%. The rate of gluconate production and duration of fermentation were monitored. These results are summarised in **Table 5.**

Table 5: Profile of fermentation as a function dose of inoculum

Inoculum dose (%)	Fermentation duration(h)	Rate of production(g/ltr/h)
5	36	4.16
10	24	6.25
15	30	5.00

From Table 5, it is clear that 5% inoculum has shown least production rate and consequently taken longest duration, whereas 10% inoculum has given optimal productivity in least duration (merely 24h) as compared to 5 and 15% inoculum. In 15% inoculum, more biomass was observed, but the conversion rate was lower. It was, therefore, considered that 10% inoculum dose (v/v) was optimum.

Age of the inoculum

For this purpose, 18, 24 and 30h old inoculum was inoculated into the production media. During the fermentation, calcium gluconate production and fermentation duration were monitored. These results are summarised in **Table 6.**

Table 6: Profile of fermentation as a function of inoculum age

Age of inoculum (h)	Fermentation duration(h)	Rate of production(g/ltr/h)
12	26	5.76
24	24	6.25
36	32	4.68

From Table 6, it is clear that 24 h old inoculum has given maximum productivity in lesser duration, whereas 12 and 36 h old inoculum has given less production and taken longer duration for fermentation, although 36 h old inoculum offered more biomass. Therefore, 24 h old inoculum was considered as optimum for the production of calcium gluconate.

pH

The pH of the production media was maintained at 5± 0.2, 6 ± 0.2 and 7 ± 0.2 with the help of pre-sterilized calcium carbonate slurry fed into the production medium. During fermentation, rate of gluconate production and fermentation duration were monitored. These results are summarised in **Table** 7.

Table 7: Profile of fermentation as a function of pH

pH of production medium	Fermentation duration (h)	Rate of production (g/ltr/h)
5 ± 0.2	36	4.16
6 ± 0.2	20	7.50
7 ± 0.2	96	1.56

From **Table 7**, it was clear that pH 6 ± 0.2 has registered maximum production as compared to pH 5 ± 0.2 and pH 7 ± 0.2. At pH 7.0, fungal biomass was found less. It was, therefore, considered that pH 6 ± 0.2 was optimum for the production of calcium gluconate.

Rate of agitation

For this purpose, the experiments were conducted at 150, 200 and 250 rpm. The rate of production of calcium gluconate and fermentation duration were monitored. These results are summarised in Table 8.

Table 8: Profile of fermentation as a function of rpm

Rate of agitation(rpm)	Fermentation duration (h)	Rate of production(g/ltr/h)
150	30	5.00
200	20	7.50
250	24	6.25

From **Table 8**, it is clear that at 200 rpm, rate of production was the highest and consequently it needed least fermentation duration as compared to 150 and 250 rpm. Therefore, 200 rpm was considered as optimum for the production of calcium gluconate.

Rate of aeration

For this purpose, the experiments were conducted at 0.5, 1.0, and 1.5 vvm (volume of air per volume of medium) and rate of production of calcium gluconate and fermentation duration were monitored. These results are summarised in **Table 9**.

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Table 9: Profile of fermentation as a function of rate of aeration

Rate of aeration (vvm)	Fermentation duration (h)	Rate of production (g/ltr/h)
0.5	36	4.16
1.0	23	6.25
1.5	28	5.35

From Table 9, it is clear that at 0.5 vvm, bio-mass increased, fermentation duration also increased and calcium gluconate production was very low. At 1.0 vvm, the production rate was increased, consequently the fermentation duration decreased. When the aeration increased to 1.5 vvm, production rate decreased and hence fermentation duration increased. It was, therefore, concluded that 1.0 vvm aeration is optimal for the production of calcium gluconate.

Temperature

Table 10: Profile of fermentation as a function of temperature

Temperature (˚C)	Fermentation duration (h)	Rate of production (g/ltr/h)
25 ± 1	40	3.75
30 ± 1	28	5.35
35± 1	45	3.33

From **Table 10**, it is clear that at 30 ± 1^0C, maximum rate of production and consequently lesser duration of fermentation. Whereas at 25±1 and 35±1^0C, fermentation duration was longer and productivity was lesser. It was, therefore, concluded that 30±1^0C as optimum for the production of calcium gluconate.

Concentration of glucose

The concentration of glucose at 5, 10 and 15% were taken for fermentation. The rate of production and fermentation duration were monitored. The results are summarised in Table 11.

Table 11: Profile of fermentation as a function of glucose concentration

Glucose concentration (%)	Fermentation duration (h)	Rate of production (g/ltr/h)
5	18	2.77
10	20	5.00
15	24	6.25

From Table 11, it is clear that 15% glucose concentration gave the maximum rate of production as compared to 5 and 10% glucose concentration. Hence concluded for the large-scale production, 15% glucose concentration was optimal. At 20% glucose concentration, insignificant increase in productivity and more residual glucose in the broth posed problems in downstream processing with poor quality final product.

Optimized protocol of calcium gluconate preparation

Thus, through the strategy of step-wise (sequential) optimization of the above-mentioned parameters, an optimized protocol for calcium gluconate production emerged, which provided higher rate of production, less duration of fermentation and consequently less energy consumption, thereby rendering it economical and eco-friendly. The optimized parameters are a) Optimal concentration of glucose 15%, b) Optimal dose of inoculum 10%, c) Optimal age of inoculum 24 hours, d) Optimal pH 6±0.2, e) Optimal rate of agitation 200 rpm, f) Optimal rate of aeration 1.0 vvm, g) Optimal temperature 30^0C.

Scale up trials in 150 liters fermenter

After devising an optimized protocol, it was scaled up to 100 liters to study the following objectives: a) reproducibility of the rate of production of calcium gluconate, b) difficulties, if any, encountered during the scale up and find its solutions, c) the reproducibility of the optimized protocol, d) techno-economic feasibility of calcium gluconate production.

Keeping the above objectives in mind, three trials were conducted, each of 100 liters. The results are summarised in Table 12.

Table 12: Profile of calcium gluconate Scale up production

S. No.	Parameters monitored	Batch I	Batch II	Batch III
1.	Duration (hours)	28.00	29.00	26.00
2.	Temperature	29.60	29.60	28.00
3.	Agitation (rpm)	196.50	194.50	200.5
4.	Aeration (vvm)	0.98	0.95	0.95
5.	Positive pressure	0.45	0.50	0.49
6.	pH	6.20	6.00	5.96
7.	RS%	0.10	0.12	0.09
8.	Calcium gluconate %	15.45	15.95	15.15

From Table 12, it is clear that 15% glucose was reduced to 0.18–0.58 % in 26-29 hours at 30 ± 1^0C, 200 ± 5 rpm, 1.0 ± 0.05 vvm at a positive pressure of 0.50 ± 0.02 kg/cm2, calcium gluconate was produced almost quantitatively.

It was found from the scale up trials that there are absolutely no problems on the scale up to 100liters. In implementing the optimized protocol, no technical difficulties encountered. Technically, down-stream processing posed no difficulty and smooth recovery of calcium gluconate was possible.

IV. CONCLUSION

From the above studies, it is obvious that glucose can be converted in to calcium gluconate, which is the need of starch industries with no technical difficulties and can be produced commercially. Calcium gluconate is in very high demand in pharmaceutical, food, feed, poultry and aquaculture industry. The conversion of glucose into calcium gluconate, is very good value addition for starch-based industries. This process therefore, has the potential to provide additional benefits to the starch industries.

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