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STUDIES IN THE PRODUCTION OF CALCIUM GLUCONATE USING LOCALLY ISOLATED STRAINS OF ASPERGILLUS NIGER

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The locally isolated strains of *Aspergillus niger* gave better yields of calcium gluconate in submerged fermentation of glucose. Calcium gluconate fermentation reached maximum 96 and 120 hr after spore inoculation of crystalline and liquid glucose media, respectively. The rates of glucose utilisation and calcium gluconate formation decreased with the increase in the concentration of glucose (20, 25 and 30%). The addition of boron (0.28, 0.57 and 0.87%) as boric acid in the medium containing 20, 25 or 30% glucose, however, increased both the glucose utilisation and calcium gluconate formation.

Gluconic acid is produced by several microorganisms. The problems associated with its production by fermentation have been reviewed. [1,2] The species of bacteria such as *Acetobacter* and *Pseudomonas*[3–5] and of mould such as *Aspergillus*[6–8] and *Penicillium*[9–10] have been studied mostly for the biosynthesis of gluconic acid or its salts on commercial scale. The basic raw materials such as commercial crystalline glucose or liquid glucose (hydrolysed starch) is abundantly available now in Pakistan. Preliminary studies of calcium gluconate production, therefore, have been carried out in shake flasks. The purpose of the present work as to select the strains of *Aspergillus niger* which are capable of producing large amount of calcium gluconate, and to study some conditions for further increasing its production.

Material and Methods

Organisms.—Five strains of *Aspergillus niger* were used for experimental purpose. Three of them WRL-14, WRL-50 and WRL-51 were independently isolated from the soil and identified as *Aspergillus niger*. The remaining two were NRRL-3 and strain No. 67.[11]

Media.—The composition of the media used for the maintenance of the culture of *Aspergillus niger* and fermentation studies are given in Table 1. Glass-distilled water was used for the preparation of agar medium M_1 and tap water was employed for fermentation medium M_2. Stock solutions of salts were prepared using glass-distilled water. All media, unless otherwise stated, were autoclaved at 121°C for 15 min. Fermentation medium M_2 was divided into three parts for sterilization (a) glucose or liquid glucose (b) $CaCO_3$ suspended in water and (3) salt solutions.

Inoculum Preparation.—Stock cultures of all strains were kept as freeze-dried conidial suspensions of *Aspergillus niger*. The mould was transferred from the stock cultures of surface cultures in 6-oz flat bottles plugged with cotton-wool and containing 15 ml agar medium M_1. The bottles were incubated at 30°C for 5–7 days. To wash off the spores the cultures were wetted with 5 ml 0.005% aqueous solution of Monoxal O.T. (dioctyl ester of sodium sulphosuccinic acid). The agar surface was washed twice with sterile distilled water and the combined washings were made up to 50 ml and shaken with glass beads to break up clumps of conidia. 1 ml of this suspension was used to inoculate 25 ml medium.

Shake-flask Culture.—For shake-flask cultures 25 ml medium M_2 including 1 ml spore inoculum was held in a 250-ml conical flask plugged with cotton-wool. The flasks were shaken on a rotary shaker, fabricated in our workshop (throw 2", 150 cycles/min). The shaker was placed in an air-conditioned room at 28°C.

Analytical Method.—Glucose as estimated by ferrocyanide reduction method, a modification

TABLE 1.—COMPOSITION OF MEDIA USED FOR THE GROWTH OF *Aspergillus niger*.

	M_1 g/l	M_2 g/l
Constituents		
Glucose	150	Variable*
Agar	20	—
$(NH_4)_2HPO_4$	—	0.388
NH_4NO_3	2.5	—
KH_2PO_4	1.0	0.188
$MgSO_4.7H_2O$	0.25	0.156
$CaCO_3$	—	26
Trace metals	mg/l	
Fe^{+++}	2.2	—
Cu^{++}	0.48	—
Zn^{++}	3.80	—

* Liquid glucose manufactured by Rafhan Maize Products Co. Ltd., Lyallpur was also used in fermentation media.

of Fujita and Iwatake.[12] For calcium gluconate assay, a determination of soluble calcium was made by precipitation of calcium as calcium oxalate and subsequent titration with 0.1 N, $KMnO_4$ Since any acid other than gluconic acid was not detected, the amount of soluble calcium in broth should be the amount of calcium gluconate produced.

Identification of Gluconic Acid.—The biosynthesis of gluconic acid in the culture broth was confirmed by paper chromatography.[13] A solvent system butanol-acetic acid–water (4:1:5 v/v) was used and chromatograms were developed by a slightly alkaline solution of 0.04% alcoholic solution of bromophenol blue.

Results and Discussion

The glucose utilisation and calcium gluconate formation by different strains of *A. niger* during the fermentation of crystalline glucose or liquid glucose were studied (Tables 2 and 3). The results show that the glucose utilisation by all the strains of *A. niger* was higher in cultures containing crystalline glucose than liquid glucose. The optimum concentrations of glucose were 15 and 20% both as crystalline and liquid glucose. Further increase in the glucose level (20, 25, 30%), either as crystalline glucose or liquid glucose, resulted in lowering the glucose utilisation. The effect was, however, more pronounced in the latter case. All strains of *A. niger* except WRL-14 were good producers of calcium gluconate and crystalline glucose proved to be the better source of carbohydrate. The strains NRRL-67 and NRRL-3 converted 80% glucose into gluconic acid during the fermentation of 15% crystalline glucose. Higher levels of glucose (20, 25, 30%) both as crystalline glucose and liquid glucose decreased the production of calcium gluconate. The lower yield may be due to (i) the deposition of calcium gluconate crystals on the mould mycelium, because of its low solubility and thus inhibiting the growth process, or (ii) agitation or aeration of the cultures, as observed visually, were affected.

Effect of Boron.—Boron is generally added to the fermentation medium to increase the solubility of calcium gluconate in order to make use of high concentrations of glucose. The data of Tables 4 and 5 show the effect of boron addition on the biosynthesis of calcium gluconate. The amount of boron added was (0.28, 0.57, 0.85%) as boric

TABLE 2.—PRODUCTION OF CALCIUM GLUCONATE, FIVE DAYS AFTER SPORE INOCULATION, BY DIFFERENT STRAINS OF *Aspergillus niger* FROM CRYSTALLINE GLUCOSE.

Strain	15%		20%		25%		30%	
	Glucose consumed g/l	Ca gluconate g/l	Glucose consumed g/l	Ca gluconate g/l	Glucose consumed g/l	Ca gluconate g/l	Glucose consumed g/l	Ca gluconate g/l
NRRL.-3	140.0	101.7	165.2	97.0	179.0	70.0	248.0	124.0
W.R.L.-14	132.5	21.5	83.0	53.81	105.0	36.8	83.2	50.1
W.R.L.-50	142.0	72.6	180.0	112.0	216.0	116.7	223.0	117.2
W.R.L.-51	140.0	81.9	175.0	111.6	184.0	112.4	210.0	118.0
A.N.-67	134.0	109.7	170.0	53.8	125.0	63.84	133.0	48.9

TABLE 3.—PRODUCTION OF CALCIUM GLUCONATE, FIVE DAYS AFTER SPORE INOCULATION BY DIFFERENT STRAINS OF *Aspergillus niger* FROM CRYSTALLINE GLUCOSE IN THE PRESENCE OF BORON.

Strain	Glucose added (with boron)					
	20%		25%		30%	
	Glucose consumed g/l	Ca gluconate g/l	Glucose consumed g/l	Ca gluconate g/l	Glucose consumed g/l	Ca gluconate g/l
NRRL.-3 ..	172.2	103.1	182.8	104.25	253.0	168.0
W.R.L.-14 ..	133.0	72.6	119.8	42.8	157.2	108.0
W.R.L.-50 ..	195.1	115.4	229.8	119.2	273.1	162.41
W.R.L.-51 ..	182.5	113.7	244.8	114.3	268.0	145.12
A.N.-67 ..	72.8	54.0	172.8	73.24	183.0	65.3

TABLE 4.—PRODUCTION OF CALCIUM GLUCONATE, FIVE DAYS AFTER SPORE INOCULATION BY DIFFERENT STRAINS OF *Aspergillus niger* FROM LIQUID GLUCOSE.

Strain	15%		20%		25%	
	Glucose consumed g/l	Ca gluconate g/l	Glucose consumed g/l	Ca gluconate g/l	Glucose consumed g/l	Ca gluconate g/l
NRRL.-3 ..	120.0	104.0	141.0	119.0	150.0	106.6
W.R.L.-14 ..	48.0	41.23	48.0	25.2	113.0	95.5
W.R.L.-50 ..	90.0	82.96	82.0	64.45	150.0	103.0
W.R.L.-51 ..	105.0	98.64	120.0	84.0	155.0	110.2
A.N.-67 ..	50.0	36.86	102.0	62.0	141.3	80.4

TABLE 5.—PRODUCTION OF CALCIUM GLUCONATE, FIVE DAYS AFTER SPORE INOCULATION BY DIFFERENT STRAINS OF *Aspergillus niger* FROM LIQUID GLUCOSE IN THE PRESENCE OF BORON.

Strain	20%		25%	
	Glucose consumed g/l	Ca gluconate g/l	Glucose consumed g/l	Ca glu conate g/l
NRRL.-3	144.0	123.75	215.0	130.5
W.R.L.-14	60.0	39.28	195.2	134.2
W.R.L.-50	143.0	109.72	175.3	125.1
W.R.L.-51	130.0	102.12	190.1	134.6
A.N.-67	123.0	71.72	170.4	91.35

acid to the medium containing (20, 25, 30%) glucose. The utilisation of glucose, hence calcium gluconate formation, by all strains of *A. niger* was significantly increased as compared with the cultures without boron described above. It follows that the cause of lower glucose consumption in media containing higher concentration of glucose may be due to the deposition of calcium gluconate crystals in the mould mycelium.

Rate of Calcium Gluconate Formation.—The rate of calcium gluconate formation and glucose utilization by all strains of *Aspergillus niger* was studied. The amount of glucose added in the media was 20% (Figs. 1 and 2) and 25% (Figs. 3 and 4) and the sources of carbon were crystalline glucose and liquid glucose, respectively. With the crystalline glucose in the medium both the calcium gluconate formation and the glucose utilization reached maximum 96 hr after inoculation with spores. The strains of *Aspergillus niger* WRL-51, WRL-50 and NRRL-3 produced large amount of calcium gluconate in both media containing crystalline glucose and liquid glucose.

The rate of glucose utilisation in the medium containing liquid glucose, as source of carbon,

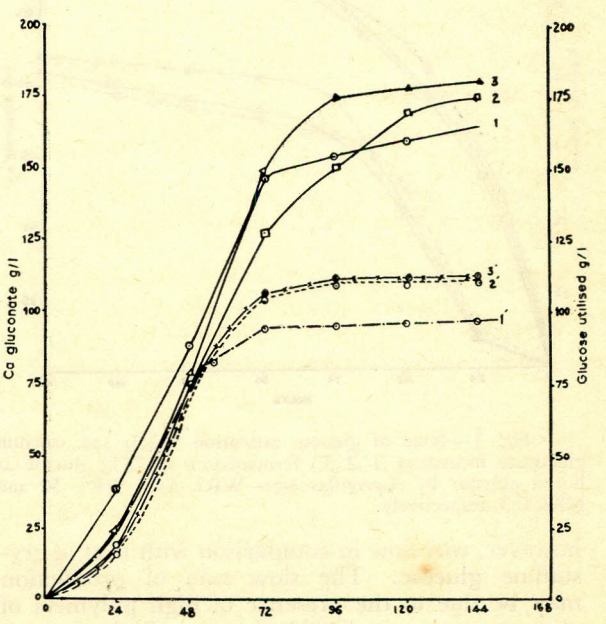

Fig. 1.—Rate of glucose utilization (1,2,3) and calcium gluconate formation (1′,2′,3′) during fermention of 20% glucose medium by *Aspergillus niger* NRRL 3, WRL 50 and WRL 51, respectively.

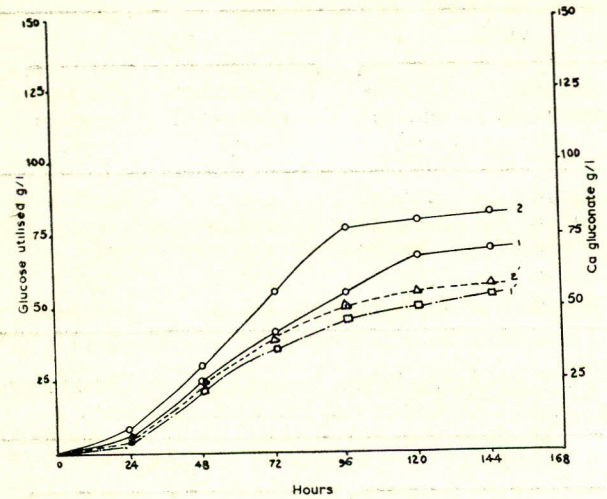

Fig. 2.—Rate of glucose utilisation (1,2) and calcium gluconate production (1′,2′) by *Aspergillus niger* A.N. 67 WRL, 14 respectively. The amount of glucose was 20% in the medium.

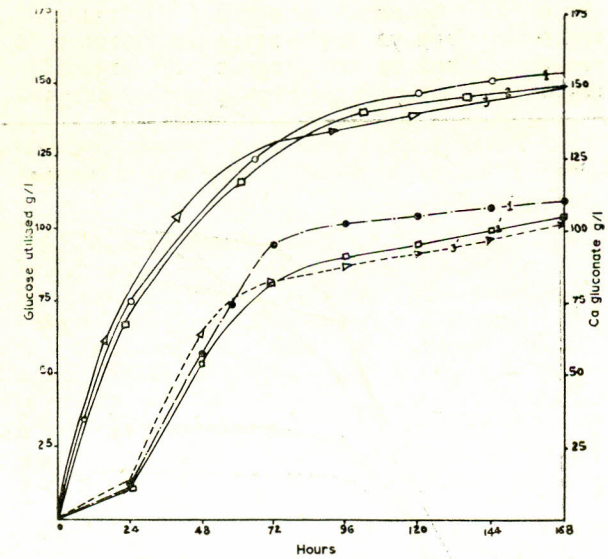

Fig. 3.—Rate of glucose utilisation (1,2,3) and calcium gluconate formation (1′,2′,3′) fermentation of 25% glucose as liquid glucose by *Aspergillus niger* WRL 51, WRL 50 and NRRL 3, respectively.

however, was slow in comparison with that of crystalline glucose. The slow rate of production may be due to the presence of high polymers of glucose present in liquid glucose. Both the calcium gluconate formation and glucose utilization reached maximum 120 hr after inoculation with spores.

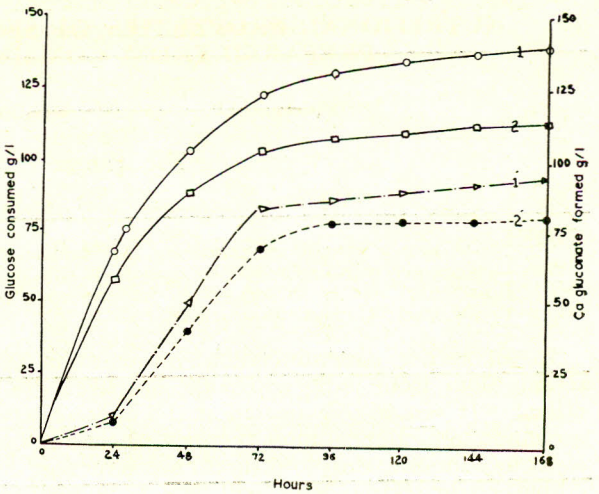

Fig. 4.—Rate of glucose utilisation (1,2) and calcium gluconate formation (1′,2′) during fermentation of 25% liquid glucose by *Aspergillus niger* A.N. 67 and WRL 14, respectively.

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