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ALANINE SYNTHESIS BY IMMOBILIZED CORYNEBACTERIUM DISMUTANS CELLS

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SUMMARY

Viable or non-viable cells of *Corynebacterium dismutans* synthesised ^{14}C-alanine using ^{14}C-glucose as its carbon source and ammonia as its nitrogen source under aerobic or anaerobic conditions. The viable or non-viable cells preparations were immobilized using several methods; both preparations immobilized by entrapment in a polyacrylamide gel matrix were thermostable and active in synthesis of alanine.

INTRODUCTION

A new species of *Corynebacterium* was isolated from Limbourg type cheese by Onyembe (1976). The cells are Gram-positive and Coryneform while the culture is in exponential phase of growth but form coccoid cells as the culture reaches its stationary phase. The species has been named as *C. distumans* due to its dismutatic property on tyrosine which resulted to the formation of *p*-hydroxyphenylpropionic and *p*-hydroxyphenyl acetic acids through intramolecular oxido-reductive reaction of tyrosine (Onyembe (1976). In a previous investigation (J.M. Sarkar) unpublished) concerned with the production of organic acids by viable or non-viable cells of *C. dismutans* supplied with glucose and ammonia as carbon and nitrogen source, it was observed that alanine was a principal metabolite. Recently Yamada *et al.* (1973) and Kinoshita and Tanaka (1972) have reported the production of L-glutamic acid and alanine in the culture medium of *Corynbacterium glutamicum* and *Corynebacterium fascians*. However, production of alanine by immobilized methods using viable and non-viable cells has not been studied.

This investigation describes the synthesis of ^{14}C-alanine by both free and immobilized cells. Cell preparations were immobilized by various methods such as adsorption on DEAE cellulose or on Kappa Carrageenan, or entrapment into polyacrylamide gel matrices.

METHODS

The bacteria were grown under aerobic or anaerobic conditions in a medium containing peptone 0.5% (w/v) and yeast extract (Merck) 0.3% w/v for 72h at 25°C. The cells were collected by centrifugation (25,000 r.p.m) washed several times with 10mM phosphate buffer (pH 7.0), lyophilized and washed several times again with dry acetone at -70°C on a porus glass filter. The dried cells were stored at -25°C. ^{14}C-alanine was synthesised in 3.0ml sterile 10mM phosphate buffer (pH 7.0) containing 60mg cells, 30mg glucose supplemented with 15µCl ^{14}C-glucose as carbon source and 30mg ammonium salt as nitrogen source. After incubation at 25°C for 24h the mixture was centrifuged (20,000

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r.p.m), the pH of the supernatant adjusted to neutrality and the clear solution passed through a cation exchanger (Dowex $50H^+$ form) column which was then washed with distilled water in order to remove all neutral and organic acid fractions. The amino acid fraction was then eluted with 1.5M NH_4OH. The eluted solution was evaporated in vacuo and the residue solubilized in 2.0ml 50% (w/v) ethanol; one portion of this solution was deposited on a planchet dried and its radioactivity was measured by Geiger Müller counter (Nuclear Chicago) in order to ascertain the quantity of ^{14}C-glucose utilized for ^{14}C amino acid synthesis from the total ^{14}C-glucose added in the reaction mixture; the remaining portion of the solution was used for two dimensional radiochromatography. The radioactivity of each separated amino acid was then measured by Geiger Müller counter.

Immobilization Procedures.

Entrapment into Polyacrylamide gel.

One hundred mg of cells (viable or non-viable) were suspended in 3.0ml of 50mM phosphate buffer (pH 7.5) and the identical procedure as reported by Chibata *et al.* (1973) was used.

Immobilization on Kappa-Carrageenan.

One hundred mg of cells (viable or non-viable) were immobilized using the identical procedure as reported by Tosa *et al.* (1979).

Immobilization on DEAE (DE 52) Cellulose.

(17g) DEAE cellulose were soaked in 30ml of 10mM NH_4Cl/NH_4OH buffer (pH 9.0. After 60 min, the fine particles were removed simply by decantation. A hundred mg lyophilized non-viable cells were suspended in 5ml same buffer and mixed with DEAE cellulose slurry by gentle agitation. The washed enzyme-cellulose complex was then packed in a column (12cm L, 2cm Ø).

The three processes outlined in the preceding paragraphs were continued for a week, the effluent of each column reduced under vacuum and the ^{14}C-alanine was recovered as indicated above. The radioactivity of this ^{14}C-alanine was measured and the percentage of synthesis was expressed in terms of ^{14}C-glucose utilized. The ^{14}C-alanine synthesis was also verified by radiochromatography.

RESULTS AND DISCUSSION

The *Corynebacterium dismutans* is a facultative anaerobe unlike the aerobic coryneform bacteria isolated by Petros and Lynch (1975). The yield of lyophilized cells obtained under anaerobic growth was higher (140mg l^{-1}) than under aerobic growth (68mg l^{-1}) pH of 7.0 and 25°C were found optimal for maximum growth. Exhaustive washing with cold (-70°C) dry acetone assures complete dehydration and non-viability of the viable cells. The synthesis of ^{14}C-amino acids was confirmed by radiochromatography (Fig.1), where among the total amino acids synthesised, the ^{14}C-alanine synthesis was predominant (85%) and the remaining 15% of the synthesis consisted of ^{14}C-aspartic and ^{14}C-glutamic acids.

The effect of different (pH) buffers and variations in the ratio of cell : glucose (c) : NH_4NO_3(N) showed (Table I) that highest yield (13%) were obtained with cell glucose ratio 4:1, replacing NH_4NO_3(N)

by 10mM NH_4Cl/NH_4OH (pH 9.0).

TABLE 1. Effect of Different Buffers and Variations in Cell:Glucose: NH_4NO_3 Ratios on the Synthesis of ^{14}C Alanine.

Buffers	Ratios	Net ^{14}C Alanine synthesised %
Citrate pH 3	2:1:1	1.1
Citrate pH 4.5	2:1:1	1.7
Phosphate pH 7	2:1:1	2.0
Phosphate pH 8	2:1:1	3.2
Tris HCl pH 9	2:1:1	5.4
Tris HCl pH 9	4:1:1	10.5
NH Cl/NH_4OH pH 9	4:1:0	13.0

Effect of concentration of acrylamide monomer and BIS.

The effect of acrylamide monomer and BIS on immobilization of non-viable cells was investigated (Table 2). When 0.35g of acrylamide monomer and 0.020g of BIS were used in 3.0ml of reaction mixture, the yield of ^{14}C-alanine synthesis was maximal (7.2%).

TABLE 2. Effect of monomer concentration on immobilization of non-viable cells.

Monomers		Immobilized cells
Acrylamide (g)	BIS (g)	Net ^{14}C Alanine synthesised %
4	0.138	2.7
1.5	0.36	3.4
0.75	0.040	4.5
0.35	0.020	7.2

Comparison of the properties of free and immobilized non-viable cells.

(i) Time. The synthesis of ^{14}C-alanine by free non-viable cells as well as immobilized non-viable cells was time dependent. However, more time was required to obtain maximum yield (13%) using free non-viable cells (24h to 48h) than was required to obtain maximum yield (7.2%) using immobilized non-viable cells (9 to 12h).

(ii) Temperature. The optimum temperature for ^{14}C-alanine synthesis was 37°C for free non-viable as well as for immobilized cells.

(iii) Thermostability. The alanine synthesising capacity of viable cells, non-viable cells and immobilized non-viable cells was compared at elevated temperatures for 60 min (Fig.2). The immobilized non-viable cells were markedly more stable than the free viable and free non-viable cells.

(iv) pH. The effect of pH on the synthesis of ^{14}C-alanine using immobilized non-viable cells was compared with that using free non-viable cells. Optimal pH for activation of the immobilized non-viable cell was 7.5 whereas for free non-viable cell was 9.0.

(v) Metals. The effect of various mono and bivalent ions on the synthesis of ^{14}C-alanine was investigated using free non-viable cells and immobilized non-viable cells. The results obtained (Table 3) indicated decrease in ^{14}C-alanine synthesis when Na^+, Mn^{2+}, K^+, Mg^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+} were used.

TABLE 3. Effect of metals on ^{14}C-alanine synthesis.

Metals	% Inhibition	
	Non-viable cells	Non-viable Immobilized cell
Na^+	9	15
Mn^{2+}	15	25
K^+	19	23
Mg^{2+}	22	36
Co^{2+}	27	36
Fe^{2+}	30	38
Ca^{2+}	56	45
Cu^{2+}	62	55
Zn^{2+}	72	58

(vi) Reuse of free non-viable and immobilized non-viable cells.

The possibilities of reusing free non-viable cells and immobilized non-viable cells were examined. The results (Fig. 3) showed that free non-viable cells lost much of their ability to synthesise ^{14}C-alanine during reutilization whereas when non-viable cell immobilized with polyacrylamide gel lattice and carrageenan synthesised the ^{14}C-alanine steadily and the yield remained 7% and 4% respectively. Since the superiority of immobilized cells over free cells was confirmed, the conditions for the continuous synthesis of ^{14}C-alanine was investigated using immobilized non-viable cell columns. Three columns were packed with DEAE cellulose-polyacrylamide-and carrageenan immobilized cells and were operated constantly over a week at a flow rate below space velocity (SV)=0.1 hr^{-1}. The results (Fig. 4) showed that the non-viable cells of *Corynebacterium dismutans* can be immobilized on all these supports, but the quantity of ^{14}C-alanine synthesised varied - the entrapment into polyacrylamide gel lattice synthesise higher yield than the other two supports.

(vii) K_m and V_{max} values. The Michaelis constant (K_m) and maximum velocity (V_{max}) values were calculated for the ^{14}C-alanine synthesised using non-viable cells, cells entrapped in a polyacrylamide gel matrix, and cells immobilized on carrageenan (Table 4). Values were somewhat different in each case. The apparent increase in the K_m values of immobilized cells indicated that some inactivation of the enzyme system had definitely occurred.

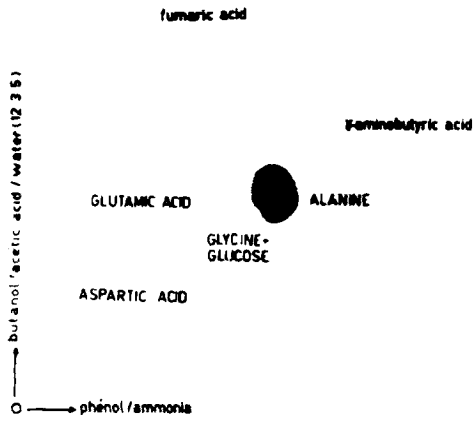

Fig. 1. Radiochromatogram of metabolites from nonviable cells.

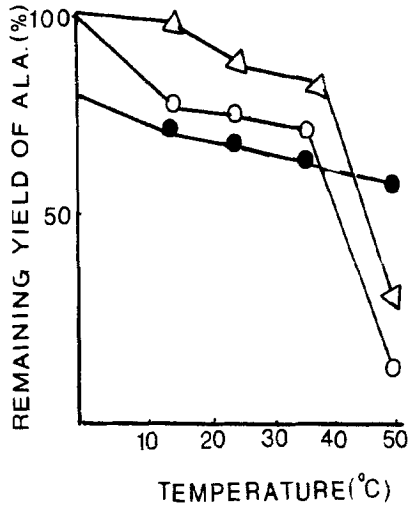

Fig. 2. Inactivation in 1 hour at stated temperature of: (Δ) viable cells; (O) nonviable cells; (●) nonviable cells in polyacrylamide gel.

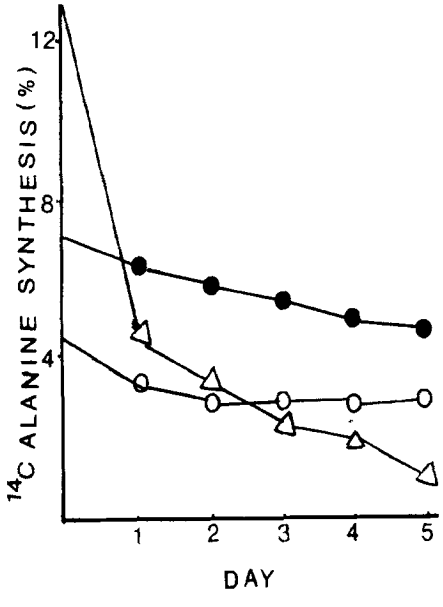

Fig. 3. Effect of daily batch re-use on nonviable cell activity: (Δ) free cells; (●) cells in polyacrylamide gel; (O) cells on carrageenan.

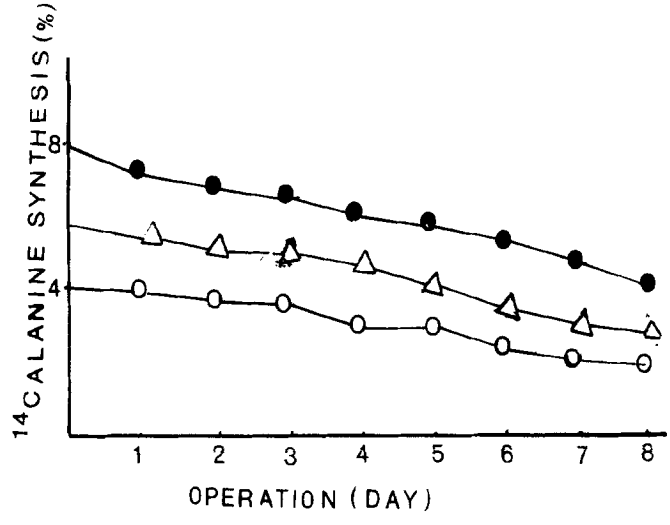

Fig. 4. Effect of continuous reactor re-use on immobilized nonviable cell activity : (●) in polyacrylamide gel; (Δ) on DEAE cellulose; (O) on carrageenan.

TABLE 4. K_m and V_{max} values for free and immobilized cells.

Samples	K_m(m-mol)	V_{max}(m-mol)
Non-viable	1.74	12.05
Non-viable cells entrapped into polyacrylamide gel	2.3	9.3
Non-viable cells immobilized on carrageenan	3.08	6.6

In conclusion, ^{14}C-alanine was synthesised from ^{14}C-glucose and nitrogen under aerobic or anaerobic incubation of non-viable cells. However, the amount of ^{14}C-glucose utilized under aerobic incubation was higher than under anaerobic incubation. This indicated that the ^{14}C-alanine synthesised from ^{14}C-glucose under aerobic incubation was synthesised by way of the citric acid cycle, and under anaerobic conditions by glycolytic pathway.

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