STUDIES ON MICROBIAL METABOLISM OF MYO-INOSITOL

1. Noncyclic Pathway of Myo-Inositol Breakdown by *Xanthomonas panici*

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Abstract

Various species of bacteria isolated from inositol enrichments have been listed and two species of *Xanthomonas*, *X. panici* and *X axonopodis*, have been pointed out to be the most active agents of inositol decomposition. Tartrate, glycolate, glyoxylate, oxalate, formate and carbon dioxide have been shown to be the breakdown products of inositol and based on chemical and chromatographic analyses and manometric experiments, a scheme for the pathway for inositol decomposition has been suggested.

Ever since Eastcott (1928) proved that Bios I is inositol, efforts have been made to determine not only the microbial requirements for inositol but also the nature of the products formed from it by micro-organisms. Ample evidence exists that numerous bacteria can attack inositol to give rise to different intermediary products and/or oxidise it to carbon dioxide (Kluyver and Boezaardt, 1939; Kluyver *et al.*, 1939; Magasanik, 1951 and 1953; Magasanik *et al.*, 1953; Volk and Pennington, 1951 and 1952). The pathway of its oxidation appears to be similar to that established for the oxidation of inositol by concentrated nitric acid by Gelormini and Artz (1930). Surprisingly, however, the use of enrichment culture methodology for the isolation of inositol decomposing bacteria was not exploited to the fullest possible extent by any of the investigators engaged in the work until Volk and Pennington (1951) explored it, but their efforts resulted in the isolation of only certain unidentified species of *Aerobacter*. On the other hand, microbiological examination of several soils (Khambata *et al.*, 1960) led us to the conclusion that the soil contained inositol decomposing bacteria other than those hitherto reported (Meillere, 1907; Weiss and Rice, 1917; Hewitt and Steabben, 1921; Kumagawa, 1922; Kluyver and Boezaardt, 1939; Kluyver *et. al.*, 1939; Magasanik, 1951 (*a*); Volk and Pennington, 1951, 1952; Kotaro Koyama, 1954; Ida Ørskov, 1955; Pickett and Nelson, 1955; Wheater, 1955; Spencer, 1955; Hajime Kodota, 1956). Therefore a comprehensive study of the soil micro-organisms decomposing inositol was undertaken in this laboratory a few years ago. This paper deals with the isolation and identification of bacteria decomposing inositol and with the determination of the metabolic products formed therefrom by *X panici*.

Materials and Methods

Enrichment and isolation of the inositol decomposing bacteria:—A medium of the following composition in *g/L* made in distilled water and adjusted to pH 7.0 admirably met the requirement of isolation of inositol decomposing bacteria : *m*-inositol, 5 ; $(NH_4)_2SO_4$, 0.5 ; $Na_2HPO_4 \cdot 12H_2O$, 0.8 ; KH_2PO_4, 0.2 ; $MgSO_4 \cdot 7H_2O$, 0.5 ; NaCl, 0.05 ; $FeSO_4.7H_2O$, 0.0025 ; $CaSO_4.2H_2O$ (Sat. sol.), 5 ml; micronutrient solution,* 1 ml.

The medium was used in 10 ml. quantities placed in 50 ml. Erlenmeyer flasks (for primary isolation) and, subsequently, in 5 ml. aliquots in $6 \times 1\frac{3}{8}''$ test tubes placed in slanting positions with a view to provide adequate surface for aeration. The inocula used were various samples of soils collected from different parts of this country. Generally speaking, good growth was evidenced within 48 hours of incubation at the room temperature (25-27°C) and after three successive transfers, inoculations were made on solid medium of the same composition and incubated aerobically. Dissimilar colonies were picked, suspended in sterile water, and streaked on second plates. If all the colonial forms on the second plate were the same, a single colony was transferred to the original liquid medium and the ability of the strain to decompose inositol determined quantitatively before recourse was taken to purify the culture further and use it for other studies. To determine the rate of decomposition of pure inositol by the bacteria, the liquid medium was dispensed in 50 ml. quantities in 250 ml. Erlenmeyer flasks and autoclaved. A loopful of cells from the slant cultures of each of the pure cultures of bacteria was inoculated into this medium and incubated at 25-27°C for eight days. An uninoculated flask provided control to check the sterility of the medium used and to determine the initial amount of inositol contained in it. Inositol was estimated by a modification of the method suggested by Fleury and Joly (1937) which was standardised to give not more than 1.5 per cent error.

Identification of bacteria decomposing inositol:—By using the keys provided in the Bergey's Manual (Breed *et al.*, 1957) and utilising the materials specified in the Manual of Microbiological Methods (Conn *et. al.*, 1957), all the bacteria isolated were separated into various genera and species. No attempts were made to prove the pathogenicity or otherwise of the species characterized as plant pathogens for their respective hosts. Table I presents the list of species, number of strains under each species and the amount of inositol decomposed by them in four and eight days respectively. A graphical representation of the maximal percentage of inositol decomposed in 1, 2, 4 and 8 days is shown in Figure 1.

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* Per L. dist. water. $ZnSO_4.7H_2O$, 11 g; $MnSO_4.H_2O$, 5 g; $CoSO_4$, 0.05 g; H_3BO_3, 0.05 g; $Na_2MoO_4.2H_2O$, 2 g; $CuSO_4.5H_2O$, 0.007g.

TABLE I

Decomposition of inositol by bacteria isolated from m-inositol enrichments

Bacterial species	No. of strains isolated	%Inositol decomposed in 4 days (Min.)	(Max.)	8 days (Average)
Xanthomonas panici	8	38.9	46.9	69.8
Xanthomonas axonopodis	10	38.8	43.3	59.1
Paracolobactrum aerogenoides	7	40.8	44.4	57.5
Pseudomonas vitiswoodrowii	16	10.0	28.3	32.5
Pseudomonas helianthi	5	20.0	26.6	26.6
Pseudomonas perolens	2	40.8	46.9	58.5
Pseudomonas maublancii	1		20.1	20.1
Flavobacterium fucatum	1		17.0	17.0

Substrates utilised for growth:—The ability or otherise of the representative strains of the bacteria to utilise a variety of organic materials including inositol as sources of carbon was tested in the liquid medium containing ammonium sulphate as the only nitrogen source. Utilisation was judged by an increased turbidity over the control with no added substrate. Likewise, nitrogen source utilisation was tested in the presence of two different carbon sources, *viz.*, glucose and inositol. Provision was also made to see if growth could occur in the absence of combined nitrogen.

Effect of temperature on inositol decomposition:—Since bacteria belonging to the family *Pseudomonadaceae* exhibit preferential temperature for growth and activities, the influence of two temperatures, *viz.*, room (25-27°C) and the incubator (37°C) on the utilisation of inositol was studied. The results recorded for *X. panici*, which exemplify also for the other *Xanthomonas* species studied, *viz.*, *X. axonopodis* are graphically presented in Figure 2 (*a*).

Effect of shaking on inositol decomposition:—Since the breakdown of inositol by the *Xanthomonas* species is an oxidative process it was considered worthwhile to see if aeration by shaking of cultures had any effect on the rate of inositol decomposition. Continuous aeration was achieved by shaking the flasks (250 ml. Erlenmeyer) containing 50 ml. of inositol medium on a mechanical (reciprocal) shaker and the activity was measured by determining the the amount of inositol decomposed after 2, 4 and 8 days. The results shown in Figure 2 (*b*) are typical.

Effect of calcium carbonate and shaking on inositol utilisation:—The culture under study was grown in inositol broth under conditions of continuous shaking with and without calcium carbonate (sufficient to maintain neutral conditions throughout) for eight days. The inositol decomposed was measured in each case. The results are shown in Figure 2 (*c*).

Products of inositol and tartrate fermentation:—Fermentation liquors of inositol and tartrate incubated for four days were separated into three fractions, *viz.*, neutral volatile products, volatile and non-volatile acid fractions by employing methods described in "Analytical methods for Bacterial Fermentations" (Neish, 1952). The neutral volatile fraction was concentrated under reduced pressure and its specific gravity was determined and was found to be 1.00. Ethyl acetate, iodoform and V. P. tests were carried out to detect the presence or otherwise of alcohol and acetoin.

The amount of volatile acids was determined from the volatile acids fraction by employing standard methods. Paper chromatographic procedure of Brown and Hall (1950) was adopted for ascertaining the number of volatile acids present. Simple preliminary tests for acetic acid (ethyl acetate test), propionic acid (solubility of ferric salt in amyl alcohol) and formic acid (silver mirror test, permanganate reduction and mercuric chloride precipitation) were also carried out.

The non-volatile products were subjected to simple preliminary chemical tests, *viz.*, calcium chloride precipitation, solubility of calcium salt in dilute acetic acid, silver mirror test, permanganate decolourisation, Fenton's test, resorcinol test, etc.

The circular paper chromatographic procedure of Giri *et. al.* (1953) was modified as follows to obtain reliable information on the products of inositol breakdown.

1. A thicker wick was used so as to finish the running of the chromatogram in less than three hours.

2. Solvent mixture recommended, *viz.*, n-butanol-formic acid-water (15-10.5-15) was kept in the separating funnel for 24-27 hours instead of the recommended 10 hours. The lower layer of free formic acid was repeatedly removed (at least 4-5 times), the first removal being achieved after 20 hours. Free formic acid layer was never used for saturating the chromatographic chamber.

3. The chromatograms were dried for two days at room temperature and for four days at 70-75°C. Hot air was then blown over them for 15 minutes.

4. The spraying reagent used was bromophenol blue of pH 6.5 instead of recommended bromocresol green of pH 5.5.

Acids were observed as yellow spots in deep to pale blue background. With all these modifications it was found that the central formic acid spot

which always developed) could not be completely eliminated. However, its size could be limited to an insignificant spot (Figure 6) and reasonably beyond the scope of vitiating the results. This procedure was found satisfactory and was therefore used in the identification of non-volatile products.

TABLE II

Products of inositol and tartrate breakdown by *X. panici*

Fermentation of	Product detected	How detected
Inositol	Tartaric acid	Preliminary chemical tests. non-volatile acids chromatography (Rf value = 0.50). simultaneous adaptation technique. (Stanier, 1947)
	Glycolic acid	Preliminary chemical tests. test with 2 – 7 – dihydroxy naphthalene . non volatile acids chromatography (Rf value = 0.10). simultaneous adaptation technique.
	Oxalic acid	Preliminary chemical tests. fusion with diphenylamine·non volatile acids chromatography (Rf value = 0.22). simultaneous adaptation technique.
	Glyoxylic acid	Simultaneous adaptation technique.
	Formic acid	Preliminary chemical tests. volatile acids chromatography (Rf value = 0.10). simultaneous adaptation technique.
	Unidentified acid	Non-volatile acids chromatography (Rf value = 0.31).
Tartaric acid	Oxalic acid	Preliminary chemical tests. fusion with diphenyalamine. non-volatile acids chromatography (Rf = 0.22).
	Formic acid	Preliminary chemical tests. volatile acids chromatography (Rf = 0.10).
	Unidentified acid	Non-volatile acids chromatography (Rf = 0.33).

Certain specific tests like diphenylmine fusion test in presence of phosphoric acid (Colowick and Kaplan, 1957) and 2-7 dihydroxy-naphthalene fusion

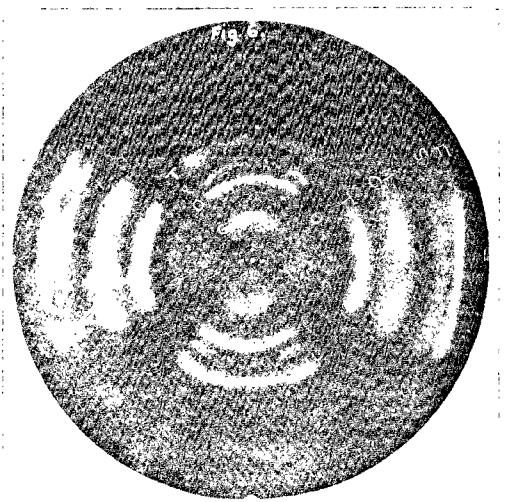


FIG. 6

Circular paper chromatogram showing the breakdown products of inositol by *X. panici* and *X axonopodis*

1. 5 μl. of standard mixture of organic acids
2. 5 μl. of concentrated products of inositol breakdown by *X. panici*.
3. 10 μl. of standard mixture of organic acids.
4. 5 μl of concentrated products of inositol breakdown by *X. axonopodis*.

F—Fumaric acid; S—Succinic acid; M—Malic acid; C—Citric acid; T—Tartaric acid O—Oxalic acid; ?—Unidentified acid. (Rf—0 31; see text); G—Glycolic acid

test (Colowick and Kaplan, 1957) were carried out to confirm the presence of oxalic and glycolic acids respectively.

The deproteinised fermentation liquors were treated with 2-4-dinitrophenyl-hydrazine reagent (Colowick and Kaplan, 1957) to determine the presence or otherwise of keto and/or aldehydic compounds.

Manometric experiments with resting cells:—Resting cell preparations were made by centrifuging off the cells from the synthetic media incubated aerobically at 25-27°C and containing 0.5 % inositol, 0.5 % tartrate and 0.5 % acetate as the carbon sources. Because of the slow growth, acetate cells were harvested after 96 hours and the others after 36-48 hours. Cells grown in glucose could not be used for the control series of experiments inasmuch as Fischer (1944-45) has postulated interconversion between glucose and inositol and, what is more, identical products were, in fact, detected by us in both the inositol and glucose fermentations. Acetate was considered preferable because of its two carbon structure and also because it did not appear to be involved in inositol decomposition even though the other 2-carbon acids were formed from inositol by these bacteria.

The harvested cells were washed thrice in M/30 phosphate buffer of pH 7.0 and resuspended in that buffer in such a way that 1.5 ml. contained 200 mg. of cells (wet wt.) before use or storage in the refrigerator. The cells once harvested were used within two hours.

Manometric measurements were carried out at 30°C in conventional Warburg vessels (Umbreit *et. al.*, 1957) using KOH papers and air as gas phase. The total volume in each vessel was 3.2 ml. The substrates, in 5 μ mole amounts, were tipped in from the sidearm of each vessel. Blanks have been subtracted from all the results presented in Figures 3, 4 and 5.

Results

It is clear from the results given in Table I that enrichment cultures with inositol as the sole source of carbon result only in the isolation of Gram negative bacteria among which species in the family *Pseudomonadaceae* predominate. *X. panici* stands out as the one which could be considered as the most efficient of the inositol decomposers. This is closely followed by another species in the genus, *viz.*, *X. axonopodis*. *Paracolobactrum aerogenoides* strains are active in inositol breakdown and appear to be identical with the *Aerobacter* species encountered by Volk and Pennington (1951) in inositol enrichments. Numerically, however, *Pseudomonas* species, notably *P. vitiswoodrowii* dominate over all other species in the genus taken together but nonetheless is poor in its power of decomposing inositol as compared to the members in the genera *Xanthomonas* and *Paracolobactrum*. It is interesting to note that strains of *Pseudomonas perolens* are occasionally encountered in the inositol enrichments

and that they are more active in inositol decomposition than are the other *Pseudomonas* species frequently associated.

The only species of *Flavobacterium* met with may be regarded as of no importance from the point of view of inositol decomposition as it proved to be comparatively poor utiliser of inositol. The finding that the majority of bacteria isolated from decomposing inositol represent species which are pathogenic for one or the other plant species and in general belong to the family *Pseudomonadaceae* suggests that plant pathogens probably exist in soil as saprophytes and infect plants under certain conditions because, among other properties, they have the ability to adapt to and decompose inositol. It however remains to be established whether most, if not all, the plant pathogenic bacteria are capable of attacking inositol.

Both species of *Xanthomonas* could utilise well inositol, mannitol and sorbitol but whereas *X. panici* was able to grow very well in the presence of dulcitol, *X. axonopodis* failed to do so. Neither species could use methanol or ethanol. Glucose, dextrin and starch were well fermented by the cultures to produce acid. Though oxalate could not serve as a source of carbon, it had no inhibitory effect on the utilisation of other salts of organic acids. The anions well utilised by both the species were: succinate, malate, fumarate, lactate and tartrate, and less so, formate, acetate and citrate. Benzoate could be used only by *X. panici*.

Generally, slight growth occurred even in tubes which did not contain any added nitrogen. Sodium ammonium hydrogen phosphate (Microcosmic salt) and nitrate were not favourable as nitrogen sources, though slow growth was evidenced in their presence. Ammonium salts appeared to serve as better sources than organic nitrogen when inositol was the carbon compound available, while organic nitrogen (glutamate and peptone) proved to be the better source when glucose was the carbon compound used. In general it may be stated that glutamate and peptone are the preferred sources for nitrogen.

Temperature, on prolonged incubation, it would seem from Figure 2 (*a*) had little effect on the rate of inositol decomposition by the *Xanthomonas* species though it had the effect of hastening inositol decomposition during the shorter period of incubation. Oxygen, which may well be expected to take an active part, had indeed, a pronounced influence on inositol breakdown, not only in the case of the *Xanthomonas* species under discussion but also in the case of *X. axonopodis* and two other *Pseudomonas* tested [Figure 2 (*b*)]. *X. panici* brought about a quantitative breakdown of inositol within 8 days under conditions of continuous shaking when sufficient calcium carbonate had been added to maintain neutral conditions [Figure 2 (*c*)]. This indicates that acidity produced is the cause of cessation of further growth.

Alcohol and acetoin did not accumulate either in inositol or tartrate fermentations. Likewise, neither acetic acid nor propionic acid could be detected on the chromatogram or in the chemical tests conducted for the purpose. Furthermore, the culture took a long time even to get adapted to

and on adaptation grew poorly in acetate. Formate, on the other hand, could be spotted chromatographically as well as chemically in both the liquors (RF = 0.10) and served well as a growth substrate.

Non-volatile acid chromatography of inositol liquor revealed four spots, (Figure 6). Three of them were identified as glycolic acid spot, oxalic acid spot and tartaric acid spot. The fourth spot occupied a position midway between oxalic and tartaric acid (Rf. 0.31). Tartrate liquor revealed the oxalic acid spot and the unidentified spot with the same RF(Rf = 0.33) which could not be recognized. Chemical tests also pointed to the presence of these compounds. Thus the metabolic products from inositol could be identified as formic, glycolic, oxalic and tartaric acids. Products of tartrate breakdown presumably were formic and oxalic acids (Table II). Both the liquors gave distinct coupling reactions with diphenylamine thereby confirming the accumulation of oxalic acid. Inositol liquor gave a distinct positive reaction with 2-7 dihydroxy naphthalene, thereby confirming the presence of glycolate. The acid giving an intermediary spot between oxalate and tartrate was spotted in both the fermented liquors. Oxalacetate, suggested by Shilo (1957) and Shilo and Stanier (1957) to be the one involved in tartrate breakdown by certain *Pseudomonas* species, may be ruled out here as it did not give a similar spot.

Since the fermented broths at all stages revealed only traces of keto or aldehydic compounds, it is unlikely that the pathway of metabolism in this case proceeds on the lines suggested by Kluyver *et al.* (1939) for *Pseudomonas beijerinckii*. Orcinol reacting substances were, however, detected in small quantities but in all probability these were the resultant products of cell autolysis.

The results represented in Figure 5 clearly prove that cells of *X. panici* (similar figures have been obtained for *X. axonopodis*) adapted to acetate could utilise oxygen, without any lag phase, in the presence of this substrate although they could not do so in the presence of inositol, tartrate, glycolate, glyoxylate, oxalate and formate. Inositol adapted cells (Figure 3), on the other hand, showed evidence of immediate oxygen uptake not only in the presence of inositol but also its postulated intermediates, tartrate, glycolate, glyoxylate, oxalate and formate, thus lending support to the view that all these compounds are in the pathway of inositol oxidation.

Inositol adapted cells, however, could not utilise *dl*-epi-inosose at all and could utilise 2-keto-myo-inosose only after a lag period of 20 minutes. The results thus suggest that *dl*-epi-inosose and 2-keto-myo-inosose are not involved in the inositol breakdown.

Discussion

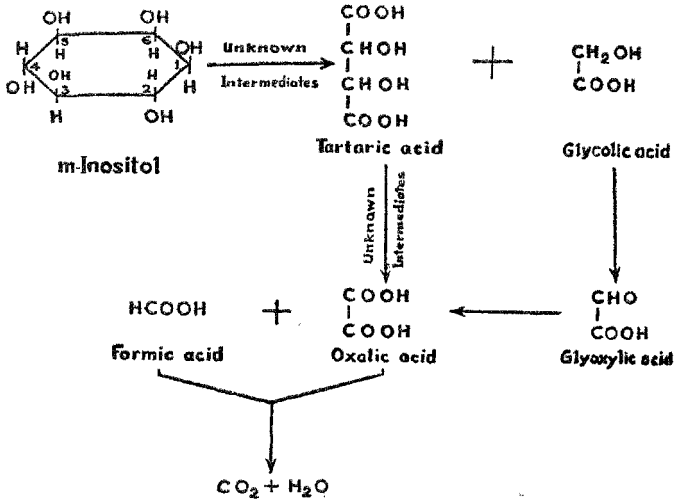
Two points of interest derived from these experiments may be considered. Inositol enrichments not only result in the isolation of species of two genera of bacteria previously unkown to be associated with its degradation but have

revealed the dominant part played by the species in the genus *Xanthomonas*, and (2) whereas (previously) the bacterial pathways suggested for the breakdown of inositol included such cyclic compounds as 2-keto-myo-inosose and L-1-2-diketo-inositol as intermediates, the pathway herein proposed rules them out and takes account of only the noncyclic compounds as intermediates in the inositol metabolism. It is significant to note that the inositol adapted cells could not utilise either the 2-keto-inositol or *dl*-epi-inosose without a considerable lag phase. What is more, neither of these substances were detected at any stage of inositol decomposition by this organism. It is therefore very unlikly that these are involved in the metabolism of inositol as shown for other bacteria (Kluyver and Boezaardt, 1939; Kluyver *et. al.* 1939; Magasanik, 1951 (*a*) and (*b*), 1953 (*a*) and (*b*); Volk and Pennington, 1951 and 1952. Since 2-keto-inositol does not seem to be involved, it is more than likely that inositol ring gets cleaved to yield a 4-carbon and a 2-carbon skeleton, which presumably, in this instance, go to form tartaric and glycolic acids, an observation similar to that reported by Butkevich and Melnikova (1943) in the breakdown of glucose by *Aspergillus niger*. It may be pointed out that permeability factors interfering in the manometric experiments do not seem to be involved herein as the parent molecules of inositol are not in the least inhibited by cell permeability factors.

Although the stepwise degradation of inositol has not been worked out here, there is some evidence which goes to show that tartaric and glycolic acids constitute the primary products. Tartaric acid, as has been indicated in Table II, appears to give rise to oxalic acid and thereafter formic acid. Glycolic acid, it would appear, is the precursor of oxalic acid, presumably *via* glyoxylate. Some of these reactions have in fact been observed in bacteria similar to the one under study here (Jakoby and Bhat, 1958). Obviously oxalic and formic acids are finally oxidised by these aerobic species to carbon dioxide and water. Thus the evidence presented here justifies postulatation of the scheme (*vide* opposit page) to explain the metabolism of inositol by *Xanthomonas panici*.

The observed facts, *viz.*, (1) a really low rate of endogenous respiration, (2) a considerably high rate of respiration in the presence of inositol, tartrate and other intermediaries postulated, (3) a highly enhanced utilisation of acetate—a product not detected and hence not postulated in the scheme—against depressed or nil utilisation of other postulated substrates by acetate adapted cells indeed tend to support the mechanism suggested.

SCHEME



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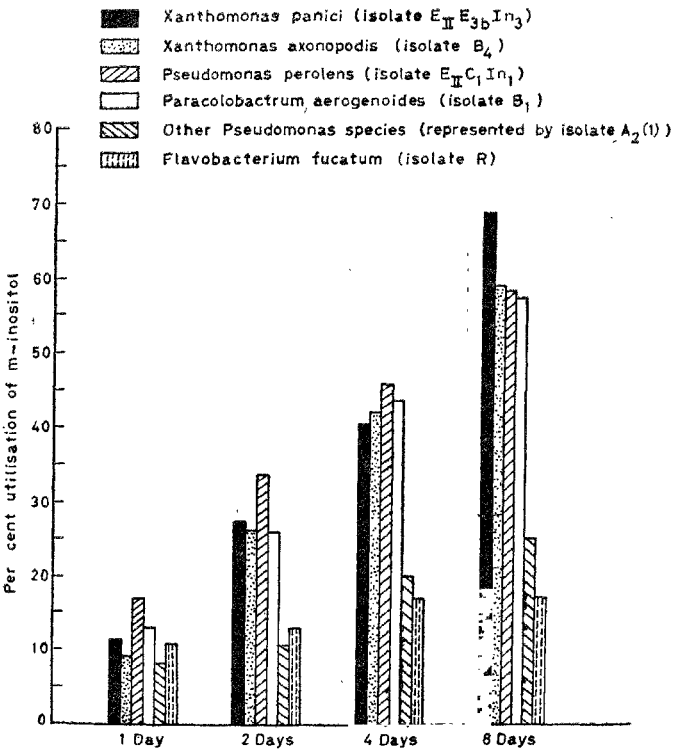

FIG. 1
Utilisation of inositol by various bacterial species

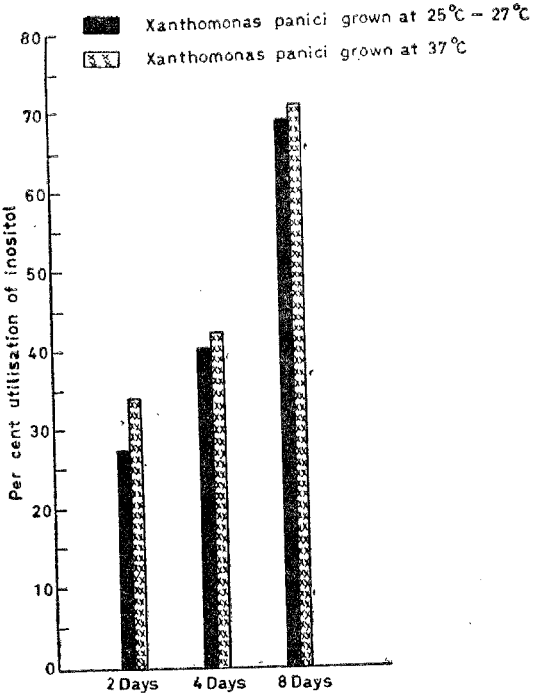

FIG. 2 (*a*)
Effect of temperature on inositol utilisation by *X. panici*

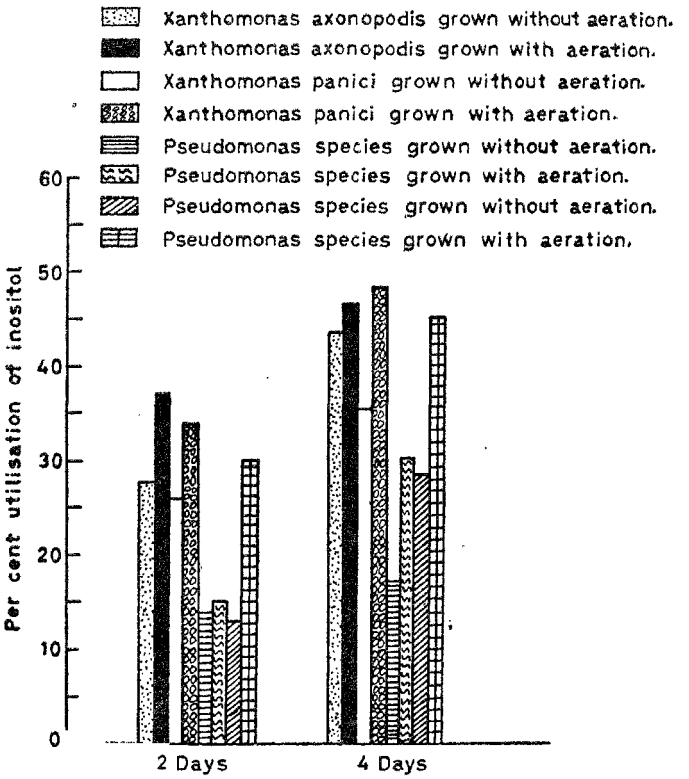

FIG. 2 (*b*)

Effect of aeration (shaking) on inositol utilisation by various bacterial species

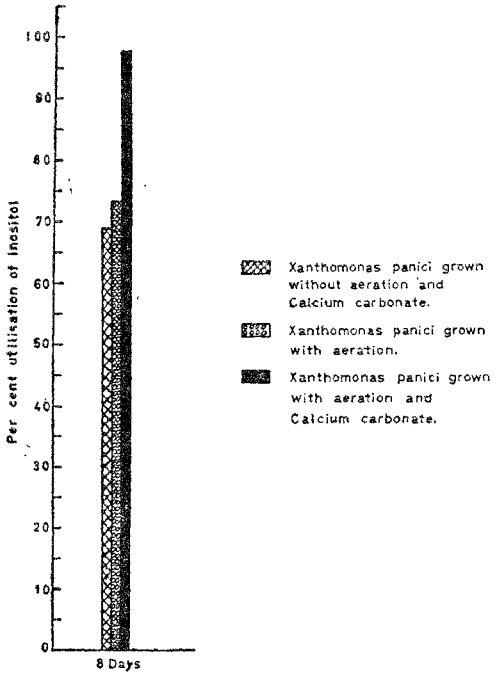

FIG. 2 (*c*)

Effect of aeration (shaking) and calcium carbonate on inositol utilisation by *X. panici*

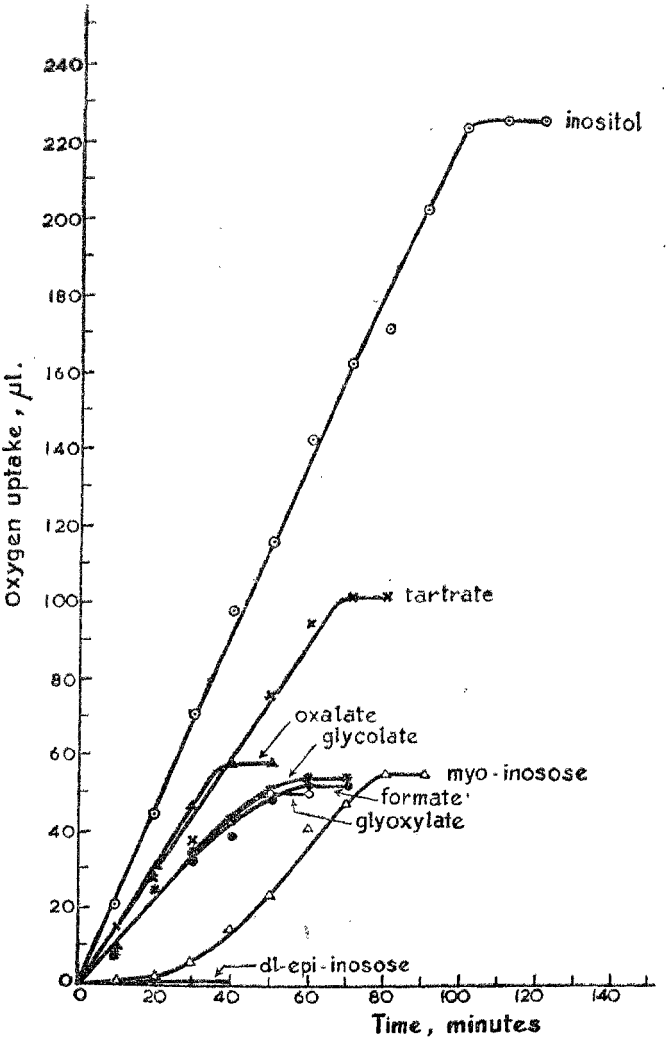

FIG. 3

Oxygen uptake from the oxidation of inositol, tatrate, oxalate, glycolate, myo-inosose, formate, glyoxylate and *dl*-epi-inosose by washed intact cells of *X. panici* grown on inositol.

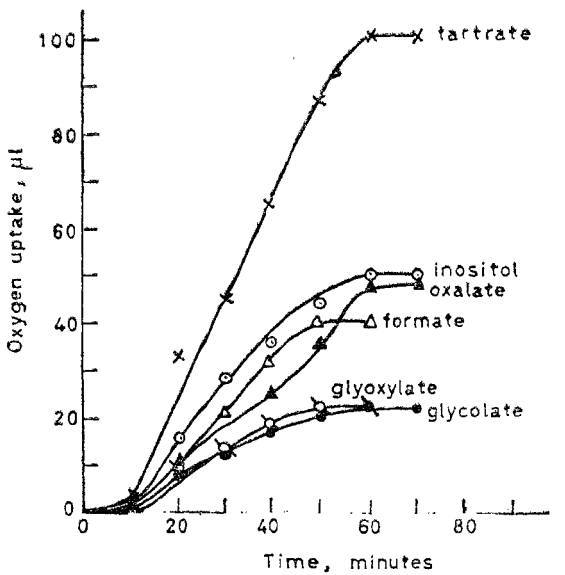

FIG. 4

Oxygen uptake from the oxidation of tartrate, inositol, oxalate, formate, glyoxylate and glycolate by washed intact cells of *X. panici* adapted to tartrate

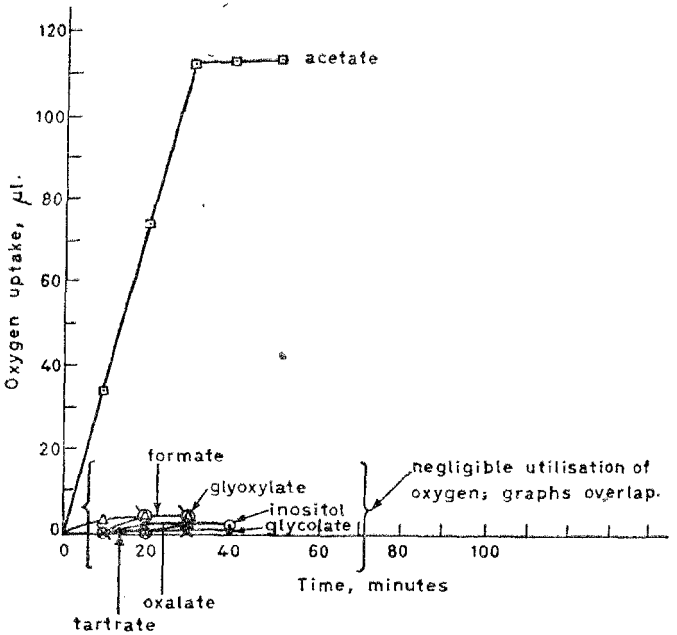

FIG. 5

Oxygen uptake from the oxidation of acetate by washed intact cells of *X. panici* adapted to acetate. Note the nil or negligible oxygen uptake from the oxidation of all other substrates.