STUDIES ON SOIL BACTERIA DECOMPOSING GLYCEROL

Part I: Isolation, identification and test for glycerol decomposition by different bacteria

Y. I. SHETHNA AND J. V. BHAT

(Fermentation Technology Laboratory, Indian Institute of Science, Bangalore-12)

Received on October 23, 1961

ABSTRACT

The results of screening 319 cultures representing bacteria, yeasts and actinomycetes for their ability to decompose glycerol have been presented. The capacity to attack glycerol has been shown to be inherent in the actinomycetes and fairly widespread in several genera of bacteria. Activity in yeasts has been shown to be somewhat limited. *Arthrobacter*, *Azotobacter* and *Pseudomonas* have been demonstrated to be the most powerful genera, among bacteria, with respect to oxidation of glycerol.

INTRODUCTION

Glycerol finds its way into soil as a degradation product of fats and oils and its removal by the scavenging microflora of soil is therefore of considerable scientific interest. No doubt literature refers to many species or organisms as possessing the power to attack glycerol and fungi as a group, it would appear, is very actively associated with the function[1]. However, no systematic effort seems to have been made either to assess the relative ability of different organisms to decompose this substrate or to compare the merit of routine culture methods and enrichment culture technique for isolating such organisms. In this paper are described the results of attempts made in this direction.

Preliminary work carried out showed[2] that species of bacteria recognisable as those of *Arthrobacter* get enriched in the presence of not only glycerol but some other substrates as glycine, asparagine, hippurate and starch. Subsequent work of Advani and Iyer[3] and Iyer *et al*[4]. brought out more clearly the morphological and other characteristics of some members of this group. The observation[2] that soils in general support per gram not less than 10^5 cells and often as many as 10^9 to 10^{12} cells capable of utilising glycerol as the only source of carbon and energy suggested that soils are furnished in good measure with the microflora functionally active in eliminating glycerol. Therefore attempts were made to isolate from soil as well as from some other sources all the possible types of organisms decomposing glycerol. Special attention was paid to study the nature and activities of *Arthrobacter* species because, unlike other genera of bacteria, these species had not been adequately dealt with before, notwithstanding the fact that they had been recognised for the past forty years[5].

The present study is based on 319 cultures of micro-organisms of which 99 were derived from soils by resort to enrichment culture technique. The details of their isolation and characteristics have been dealt with elsewhere[2,6]. However, it is necessary to mention that for enriching glycerol decomposing bacteria, a medium containing a nitrogenous compound as well as another without any source of combined nitrogen were employed and whereas the former resulted in the isolation of diverse genera of bacteria the latter gave rise to only *Azotobacter* species. The latter finding is of particular interest here in that although glycerol has been referred to as a suitable source of carbon for the isolation of *Azotobacter*[7] there is no information in the literature on its utilisation by this group as a specific source of carbon. On the contrary, a recent review[8] brought out evidence against the ability of *Azotobacter* strains to utilise glycerol. In view of these contradictory reports, the isolation of *Azotobacter*, using glycerol as the sole source of carbon, and a study of its power to utilise glycerol was considered a fruitful project.

Materials, Methods and Results

The soils used as inocula were collected from various regions and included seven samplings made from soap factory surroundings where the spent lye containing free caustic alkali and glycerol were dumped. The pH of the soils varied from 5.0 to 8.8 and those of the soap factory environments ranged between 9 and 10. Consequently the latter soils were found to be free from bacteria flourishing under normal conditions, though a few pseudomonads were encountered in glycerol-ammonium salt enrichments and obtained in pure cultures for further study. All other soils proved to be suitable inocula and permitted the growth and isolation in glycerol-ammonium salt of several species and genera of bacteria. Besides soils, a sample of rancid butter was tried as an inoculum for isolating glycerol decomposing organisms inasmuch as rancidity production is associated with many changes including the breakdown of glycerol. This attempt resulted in the isolation of only one yeast species.

For obtaining information on the relative ability to decompose glycerol by *Arthrobacter* species isolated on routine media, 17 other strains derived from soils by Advani and Iyer[3] were included in this study. Similarly, as the very object of the investigation was to gain a relative picture of the ability of the different groups of organisms to decompose glycerol, 77 cultures of *Streptomyces* isolated from soil and sea water by Freitas and coworkers (obtained from the collection of Dr. Y. M. Freitas, St. Xavier's College, Bombay, to whom the authors are indebted) together with 23 marine yeasts (stock cultures of this laboratory), 41 strains of bacteria isolated from sewage in glycerol-nitrate enrichments and 62 strains obtained from pectin enrichments inoculated with sewage, soil and plant tissues, were also screened for their ability to decompose glycerol. A few nitrogen-free mannitol enrichments were in addition set up and

Azotobacter strains were isolated, the object being to ascertain the ability or otherwise of these isolates to decompose glycerol as compared to those obtained from nitrogen-free glycerol enrichments.

Test for glycerol decomposition: All the cultures were tested for their power to decompose glycerol by inoculating them into a medium made up with the basal salt solution, 0.05 per cent ammonium sulphate, 0.01 per cent yeast extract, 1 per cent glycerol and a few drops of bromocresol purple indicator. With a view to ascertain if acidity development could prevent further decomposition of glycerol, tests were also run with 0.5 per cent calcium carbonate in the medium to serve as a neutralising agent. After inoculation the medium was incubated at room temperature ($20^\circ - 30^\circ C$) for definite periods either on a rotary shaker or as stationary cultures and the contents from each growing culture was examined for the residual glycerol. An uninoculated sample of the medium served as the control and provided information of the initial concentration of glycerol in the medium.

Glycerol was estimated by the periodate oxidation method employed successfully by Viswanathan *et al*[9]. The method was found to be very simple, quite specific and fairly reliable as the results were reproducible within 2-3 per cent error. That the products of glycerol decomposition did not interfere in its estimation by this method was ascertained before adopting the method.

As has been stated earlier, in all 90 cultures of bacteria were isolated from the glycerol enrichments and 9 more were obtained from mannitol enrichments. The latter isolates were recognised to be strains of *Azotobacter*. Some of the cultures were discarded from this study as they were found to utilise glycerol rather poorly (less than 20 per cent) in the absence of calcium carbonate. No doubt determination of their power to decompose glycerol in the presence of calcium carbonate would have shown improvements but this was not considered necessary in the present context. The genera encountered, and their relative ability to decompose glycerol, are listed in Table I.

On the basis of morphological, physiological and nutritional similarities, only 5 isolates were recognised as those of *Arthrobacter*. Two of these showed evidence of fragmentation into smaller rods (coccobacteria) thus lending themselves to be classified in the fourth group proposed by Advani and Iyer[3]. All were found to be motile. While 4 displayed monotrichous flagellation, the fifth was seen to possess a lateral flagellum. Motility and flagellation in these forms, it may be recalled, have been a subject of much discussion and controversy[10], but in the present study no difficulty whatsoever was experienced either in observing the motility by the hanging drop method or in the demonstration of the flagellar arrangement by staining. However, only four of the *Arthrobacter* cultures were observed to decompose glycerol well (more than 20 per cent). One of them *viz.*, strain, EIC3Gy2 has been tentatively identified as *Arthrobacter citreus*. This strain has been studied in detail as regards its glycerol metabolism (unpublished data). All the glycerol decomposing strains were

pigmented. One strain (S_5) was lemon-yellow to buff coloured when isolated; however, on continuous cultivation on glycerol medium the pigment has changed colour to an orange-red.

Of the 17 *Arthrobacter* cultures tested from the collection of Advani and Iyer[3], 11 were observed to be efficient decomposers of glycerol. Two others were also capable of attacking glycerol though poorly, *i.e.*, less than 20 per cent; 4 cultures could not decompose glycerol at all. All these cultures, it is necessary to emphasize, were isolated by the direct method on nutrient agar and soil-extract agar and were maintained for a long time on the former medium. The fact that the majority of these cultures could decompose glycerol efficiently (Table I) is in itself an indication of the widespread occurrence of glycerol metabolizing enzymes among these bacteria.

The largest single group isolated from glycerol enrichments was, however the *Pseudomonas*. The widespread occurrence of pseudomonas in nature and their general ability to attack a vast number of carbon compounds have been well documented in literature. Considering their versatility and highly aerobic nature, it is not surprising to find this group as the most efficient in the oxidation of glycerol. A glance at Table I would clearly reveal the dominating role of this genus in the process. Similarly all the sporeforming *Bacillus* species isolated were found to be able in this respect.

The ability of the members of the *Escherichia* and *Aerobacter* to decompose glycerol is well known. However, it is of interest to record that glycerol- ammonium salt enrichments also resulted in their isolation, though their number was limited owing to the preponderance of some other genera. A picture of the relative dominance of the genera decomposing glycerol may be seen from Table I, though this is by no means very accurate inasmuch as all those which could not decompose glycerol to the extent of 20 per cent or less have not been included in the Table. Nonetheless it is very clear that next to *Pseudomonas*, *Azotobacter* is conspicuously dominant in the glycerol enrichments. Furthermore, all the *Azotobacter* strains isolated were able to decompose glycerol very well indeed in that 9 out of the 10 had the capacity to decompose over 20 per cent; the discarded strain, not shown in the Table, was also capable of decomposing glycerol to 18 per cent level. It is equally interesting to observe that the *Azotobacter* isolated from mannitol enrichments also could attack glycerol with vigour. As a matter of fact, 3 more cultures obtained from mannitol medium were excluded for reason of their disability to decompose 20 per cent glycerol although they could do so to an extent of 16-19 per cent. It would thus appear that *Azotobacter* species not only possess the ability to utilise glycerol but are actually concerned in its disappearance under natural environments. That glycerol could serve as a suitable source of carbon in nitrogen fixation has also been ascertained in this connection (unpublished data).

Significantly, all of the *Streptomyces* species tested could decompose glycerol moderately well (Table I). It is likely that further decomposition was prevented

by the limiting pH as the study was carried out in the absence of $CaCO_3$ in the medium. At any rate, that 76 of the 77 cultures could do so beyond the 20 per cent level goes to prove that glycerol is a very good source of carbon and

TABLE I

Relative ability of different genera of bacteria to decompose glycerol

Source of culture	Genera	% glycerol decomposed		
		Min.	Max.	Average
Glycerol-ammonium enrichments.	*Arthrobacter*	82	100	93 (4 strains)
	Pseudomonas	94	100	99 (13 ,,)
	Bacillus	45	100	79 (5 ,,)
	Escherichia	94	100	98 (3 ,,)
	Paracolobactrum	.....	60	60 (1 ,,)
	Aerobacter	25	100	72 (5 ,,)
	Serratia	.....	98	98 (1 ,,)
	Sarcina	.....	100	100 (1 ,,)
Routine media	*Arthrobacter*	25	100	66 (11 ,,)
Do	*Streptomyces*	23	55	38 (76 ,,)
Nitrogen-free glycerol enrichments.	*Azotobacter*	21	93	55 (9 ,,)
Nitrogen-free mannitol enrichments.	*Azotobacter*	46	100	60 (6 ,,)
Glycerol-nitrate enrichments	*Aerobacter*	25	100	61 (12 ,,)
Pectin enrichments	Unidentified	20	50	39 (8 ,,)

energy for the development of these micro-organisms. This is in agreement with earlier reports[11–15] and explains the utility of glycerol media for their isolation[15]. To conclude, it may be said that *streptomyces* represent, as a class, micro-organisms which possess inherently the capacity to decompose glycerol.

Of the 25 yeast cultures examined as many as 10 showed the ability to decompose glycerol extremely well. The identity of these cultures together with their relative capacity to decompose the substrate within 5 days is presented in Table II. The cultures which failed to attack glycerol were 7 strains of *Candida tropicalis*, and one each of *Debaryomyces subglobosum*, *Cryptococcus laurentii*, *Saccharomyces fructuum*, *S. morei*, *Torulopsis glabrata*, *T. famata*, *T. candida* and an unidentified *Torulopsis* species. The only strain isolated from rancid butter, it may be observed, was from glycerol enrichment and it possessed the capacity to utilise this carbon source.

The finding that 3 out of 4 *Debaryomyces* species tested could completely decompose glycerol from the medium is of special interest in this connection. The product of glycerol decomposition in each case was crystallisable and has tentatively been characterised as oxalic acid. Its accumulation in glycerol

TABLE II

Decomposition of glycerol by yeasts

Source of culture	Yeast species	Inoculum	% glycerol decomposed
Stock	*Debaryomyces hansenii*	Sea water	100
,, ...	*Debaryomyces nicotianae*	,,	100
,, ...	*Debaryomyces klockerii*	,,	100
,, ...	*Candida guilliermondii*	,,	99
,, ...	*Candida melibiosi*	,,	100
,, ...	*Torulopsis famata*	,,	95
,, ...	*Rhodotorula halida*	,,	75
,, ...	Unidentified	,,	92
Glycerol enrichment	Unidentified	Rancid butter	54
,,	Unidentified	Earthworm cast	97

medium was so fast and so considerable that the medium continued to remain somewhat acidic even in the presence of 0.5 per cent $CaCO_3$. Acid formation by true yeast is, however, not peculiar in this instance. Stodola[16] has cited several examples and has reported on the zymonic acid production from glucose by *Sporobolomyces salmonicolor, Cryptococcus laurentii, Debaryomyces hansenii* and *Nematospora coryli.* The present study however indicates the suitability of glycerol as a potential source for aerobic yeast fermentation and the utility of the *Debaryomyces* species for the purpose.

The 41 sewage bacteria isolated from glycerol-nitrate enrichments were, by and large, efficient glycerol utilisers in the sense that as many as 12 cultures therefrom were able to decompose over 20 per cent glycerol in the unshaken cultures as well in the absence of $CaCO_3$. Most of these cultures have been recognised as *Aerobacter* species[17]. This finding is of considerable interest when seen in juxtaposition with those genera of bacteria which get enriched in the presence of ammonium salt rather than nitrate as the nitrogen source. In other words, by skilful combination of carbon and/or nitrogen sources it would seem possible to promote overwhelmingly the growth of singled out genera of bacteria, a finding of considerable practical interest in the laboratory.

Altogether 62 bacterial cultures, isolated from pectin enrichments inoculated with soil, sewage and plant straws, were screened for their ability to decompose glycerol. Of these, only 8 cultures decomposed glycerol beyond the

arbitrarily set limit of 20 per cent, though as many as 24 displayed action on the substrate. Considering the fact that all the cultures were enriched with pectin, a carbohydrate derivative unrelated to glycerol, one may reasonably expect these weak cultures to show enhanced rate of glycerol utilisation either after an adaptation to glycerol or in the presence of $CaCO_3$. The subject deserves further attention especially from the viewpoint of characterisation of the species involved and this would be taken up in the near future. In the meantime it may reasonably be concluded that the ability to decompose glycerol is shared by several genera of bacteria, nearly all the species of *Streptomyces* and a few yeasts and that this ability can be demonstrated in the cultures irrespective of whether they were isolated on routine media by direct methods or from enrichments made with glycerol.

ACKNOWLEDGMENT

The authors wish to thank Dr. S. Bhagavantam for his interest in the work.

REFERENCES

1. Cochrane, V. W. .. Physiology of Fungi, (London, John Wiley), 1958.
2. Khambata, S. R., Iyer, V. N., Iyer, Rajul, V., Bhat, Maya, G. and Bhat, J. V. .. *Indian J. Agri. Sci.* 1960, **30**, 91.
3. Advani, Satya and Iyer V. N. .. *Proc. Nat Inst. Sci. Ind.*, 1960, **26**, 278.
4. Iyer, V. N., Khambata, S. R and Bhat, J. V. .. *Proc. Indian Acad. Sci.* 1960, **51 B**, 157.
5. Kisskalt, K. and Berend, E. .. *Centr. Bakt, Abt.* I, orig., 1918, **91**, 144.
6. Shethna, Y. I. .. Ph.D. Thesis, Bombay University, 1961.
7. Waksman, S. A. .. Principles of Soil Microbiology, (London, Balliere, Tindall and Cox.), 1932.
8. Jensen, H. L. .. *Bact. Reviews*, 1954, **18**, 195.
9. Viswanathan, C. V., Leley, V. K. and Narayana, N. .. *J. Sci. Indus. Res.* (India), 1961, (in press).
10. Khambata, S. R and Bhat, J. V. .. *Arch. Microbiol.*, 1955, **22**, 175.
11. Waksman, S. A. .. *Soil. Sci.*, 1919, **8**, 71.
12. Conn, H. J. .. *N. Y. State Agr. Expt. Sta. Bull.*, 1921, **83**, 1.
13. Cochrane, V. W. and Conn. J. E. .. *J. Bact.*, 1947, **54**, 218.
14. Pridham, T. G. and Gottleib, D. .. *Ibid.*, 1948, **56**, 107.
15. Waksman, S. A. .. The Actinomycetes, (Mass., Chronica Botanica), 1950.
16. Stodola, F. H. .. Chemical transformations by micro-organisms, (New York, John Wiley), 1958.
17. Dias, F. F. .. M.Sc. Thesis, Indian Institute of Science, 1961.