Proc. Indian natn. Sci. Acad. **B47** No. 2 pp 229–234 (1981)

In vitro Production of Non-volatile Aliphatic Acids by Fungi

K D SHARMA and K K MISHRA
Mycology Research Labour Department of Botany, Agra College, Agra 282002

(Received 6 February, 1978)

The studies on qualitative and quantitative production of non-volatile aliphatic acids (organic acids) by soil fungi has been studied in vitro using synthetic media. In all 30 fungi were selected. In all, nine acids, viz., quinic, tartaric, citric, oxalic, malic, alpha keto glutaric, malonic, succinic and fumaric acids, were found to be produced by fungi but the behaviour of acid production was variable in different fungi.

A rapid and clear separation and identification was made following radial (circular) paper chromatography with minor modifications. The technique was found superior in comparison to ascending and descending chromatographic separation of non-volatile aliphatic acids.

Key Words: In vitro **Production, Organic acids by fungi on synthetic media, Identification and radial chromatographic separation**

Introduction

The fungi are efficient producers of non-volatile aliphatic acids (organic acids) by their chemical activities on carbohydrates (Foster 1949). The carbohydrates remain available in the form of cellulose and other complex forms from plant debrises and by death of other micro-organisms in the soil. These are broken down into simpler forms by the activities of the fungi which live in the soil as a complex population. Competition occurs continuously in the soil with the result the conditions become unfavourable for the growth of several microorganisms by changing the reaction of the medium to acidic by production of inorganic (nitric sulphuric) or organic (citric, exalic, fumaric, butyric, lactic, gluconic kojic, itaconic, succinic and malonic, etc.) acids (Waksman 1952) and only resistant organisms can grow in this environment. The production of organic acids in soil by fungi has been frequently recorded (Martin 1961). Such organisms can be studied for their relative behaviour of producing such biological substances and are used as industrial strains after their domestication for the large scale production of these substances. In the present investigations the fungi isolated from the soil of Banshipaharpur (Sharma & Mishra 1977) were studied to know their relative capacity of organic acid production, and type and number of stable forms of

acids by a particular species. Since Banshipaharpur is an ecologically distinct area, it was expected to obtain some suitable species or isolates which can be utilized successfully in the industrial production of non-volatile aliphatic acids. Therefore, study of organic acid production by soil fungi has been taken to determine the relative capacity of various fungi for the production of non-volatile aliphatic acids in vitro.

Materials and Methods

Thirty fungal species isolated from the soil were selected to see their ability to produce the organic acids. Primary screening was made on the Czapek's dox agar medium by using bromothymol and bromo phenol blue as indicators. The indicators were added in the concentration of 50 mg/500 ml in the medium before sterilization. The sterilized medium was dispersed (15–20 ml/plate) in sterilized petri dishes which were inoculated by a drop of spore suspension of pure culture of fungi. The plates were incubated at 28±1°C and observations were taken after 7 days for acidic reaction by the colour change of the indicator from blue to yellow (Casida 1964). The control sets were maintained simultaneously.

The quantitative production was studied on Czapek's dox broth medium. The medium was distributed 50 ml/250 ml Erlenmeyer flask and sterilized at 12 lb pressure for 30 minutes. The medium was inoculated with 1.0 ml of spore suspension made from pure cultures of fungi separately. After 7 days of incubation at 28±1°C the culture filtrates were tested for the quantity of organic acids following titrimetric method against 0.1N NaOH solution and amount of acid produced is expressed in terms of number of ml of 0.1 N alkali solution utilized for complete neutralization of acids in sample.

For chromatographic analysis 10 ml of the culture filtrate of each fungus was dried in the petri dishes at 70°C for 36 hours so as to remove the volatile substances completely from the culture filtrate leaving yellowish brown residue which was dissolved in 2 ml of 70% ethanol.

The analysis of these samples was made on 28.0 inches diam. circular Whatman No. 1 chromatographic paper (Brown 1939). It was divided into 10 equal strips by 3 mm slits made by cutting paper leaving 1.0 inch from margin and 3.5 inches diam. of circle from the centre. The samples were spotted on each strip separately and after drying the spots were allowed to run with solvent (butanol, formic acid and water 4:1:5 V/v Lugg & Overell 1947) in glass trough. The solvent was applied from the centre of chromatogram by a paper nipple at 25–28± 1°C temperature. Prior saturation of trough was maintained with solvent. The chromatogram was taken out after required flow, air dried and sprayed with colour reagent (dimethyl yellow 25 mg+bromo phenol blue 75 mg) in 200 ml of 96% ethanol, *p*H 7.0 (Hargreaves 1956). Yellow, light pink, dark pink spots were developed on blue background of the chromatogram. Rf values of respective organic acids were calculated and compared with that of known pure organic acids solution run simultaneously on the same chromatogram.

Results and Discussion

The results on primary screening on solid media indicated that on the basis of colour reaction took place in the medium by the growth, fungi were grouped in 3 categories, i.e., I—fast, II—moderate and III—slow acid-producers. Out of the 30 fungi tested, 4, 5 and 21 sp. were fast, moderate and slow acid producing respectively (table 1).

In quantitative production, *Alternaria alternata, Aspergillus awamorii, Penicillium*

Table 1

Fungi	Screening of organic acid producing fungi			
	On solid media (Using Bromophenol blue as indicator)	Control C1	Control C2	On liquid media *Total acidity
1. *Acrophialophora fusispora*	+	No change		0.65
2. *Alternaria alternata*	+++	,,	,,	4.8
3. *A. tenuissima*	+	,,	,,	0.1
4. *Aspergillus amstelodami*	+	,,	,,	1.15
5. *A. awamorii*	+++	,,	,,	6.05
6. *A. chevalieri*	+	,,	,,	1.05
7. *A. flavus*	++	,,	,,	1.75
8. *A. fumigatus*	++	,,	,,	2.40
9. *A. japonicus*	++	,,	,,	2.40
10. *A. luchuensis*	+	,,	,,	0.50
11. *A. nidulans*	+	,,	,,	0.67
12. *A. niger*	++	,,	,,	2.40
13. *A. rugulosus*	+	,,	,,	0.10
14. *A. sulphureus*	+	,,	,,	0.80
15. *A. sydowii*	+	,,	,,	0.45
16. *A. tamarii*	++	,,	,,	2.45
17. *A. terreus*	+	,,	,,	0.40
18. *Curvularia senegelensis*	+	,,	,,	0.05
19. *Cladosporium cladosporoides*	+	,,	,,	0.01
20. *Drechslera* state of *cochliobolus specifer*	+	,,	,,	0.01
21. *Fusarium sporotrichoides*	+	,,	,,	1.15
22. *F. moniliforme*	+	,,	,,	0.01
23. *Myrothecium roridum*	+	,,	,,	0.60
24. *Penicillium admetzii*	+	,,	,,	1.23
25. *P. citrinum*	+++	,,	,,	5.05
26. *P. expansum*	+	,,	,,	0.65
27. *P. fellutanum*	+++	,,	,,	8.65
28. *P. simplicissimum*	+	,,	,,	1.35
29. *P. variabile*	+	,,	,,	0.80
30. *Penicillium* sp.	+	,,	,,	0.05

C1=medium without carbon source; C2=medium with carbon source
+=slow; ++=moderate; +++=Fast
*=acidity expressed in ml

Table 2

Fungi	Organic acids/Rf values 1 0.17	2 0.43	3 0.44	4 0.59	5 0.65	6 0.82	7 0.85	8 0.86	9 0.97	total
1. *Acrophialophora fusispora*	+	+	+	+	−	+	−	−	−	5
2. *Alternaria alternata*	−	+	+	+	−	+	−	+	−	5
3. *A. tenuissima*	−	+	−	−	−	+	−	+	−	3
4. *Aspergillus amstelodami*	−	−	−	−	−	−	−	+	−	1
5. *A. awamorii*	+	−	−	−	+	−	−	−	−	2
6. *A. chevalieri*	−	−	+	−	−	−	+	−	+	3
7. *A. flavus*	+	+	−	−	−	−	−	+	+	4
8. *A. fumigatus*	−	−	−	−	+	−	−	−	−	1
9. *A. japonicus*	−	−	−	−	+	−	+	−	+	3
10. *A. luchuensis*	+	+	−	+	−	+	−	−	−	4
11. *A. nidulans*	+	−	−	−	−	−	−	−	−	1
12. *A. niger*	−	−	−	−	+	−	−	−	−	1
13. *A. rugulosus*	−	−	−	+	−	+	−	−	+	3
14. *A. sulphureus*	−	−	+	+	+	−	+	−	+	5
15. *A. sydowii*	+	+	−	−	−	−	−	−	+	3
16. *A. tamarii*	−	−	−	−	−	+	+	−	+	3
17. *A. terreus*	−	+	−	+	−	−	−	+	−	3
18. *Curvularia senegelensis*	+	−	−	−	−	+	+	−	−	3
19. *Cladosporium cladosporoides*	+	−	+	+	−	+	−	+	−	5
20. *Drechslera* state of *Cochliobolus specifer*	+	−	−	−	−	−	−	+	−	2
21. *Fusarium sporotrichoides*	+	−	−	−	−	−	−	+	+	3
22. *F. moniliforme*	+	−	−	−	−	−	−	−	−	1
23. *Myrothecium roridum*	−	−	+	−	−	+	−	−	−	2
24. *Penicillium adametzii*	−	−	+	−	+	−	+	−	−	3
25. *P. citrinum*	+	+	−	−	+	+	−	+	+	6
26. *P. expansum*	−	−	−	−	−	−	−	−	+	1
27. *P. fellutanum*	−	−	+	+	+	−	+	−	+	5
28. *P. simplicissimum*	−	−	+	+	+	−	+	−	+	5
29. *P. vaiabile*	+	−	−	−	−	−	+	−	+	3
30. *Penicillium* sp.	−	+	−	+	+	−	−	−	+	4

1. Qunic acid; 2. tartaric; 3. oxalic; 4. citric; 5. malic; 6. Alpha ketoglutaric acid; 7. malonic acid; 8. succinic acid; 9. fumaric acid

citrinum, and *P. fellutanum* are highly active with acidity, 4.8, 6.05, 5.05 and 8.65 respectively in the broth medium.

Moderate forms are *Aspergillus flavus*, *A. fumigatus*, *A. japonicus*, *A. niger*, and *A. tamarii* with acidity 1.75, 2.40, 2.40, 2.40 and 2.45 respectively.

Third category includes: *Acrophialophora fusispora*, *Alternaria tenuissima*, *Aspergillus amstelodami*, *A. chevalieri*, *A. luchuensis*, *A. rugulosus*, *A. nidulans*, *A. sulphureus*, *A. sydowii*, *A. terreus*, *Cladosporium cladosporoides*, *Curvularia senegelensis*, *Drechslera* state of *Cochliobolus specifor*, *Fusarium sporotrichoides*, *F. moniliforme*, *Myrothecium roridum*, *Penicillium admetzii*, *P. expansum*, *P. simplicissimum*, *P. variabile* and *Penicillium* sp., which produced less amount of acidity (<1.0 ml) in the broth medium.

The results obtained by the chromatographic analysis are shown in table 2, which indicates that in all nine acids were identified from the heterogenous culture filtrates. These are *quinic*, *tartaric*, *oxalic*, *citric*, *malic*, *α-keto-glutaric*, *malonic*, *succinic* and *fumaric* acids.

It was interesting to note that variable number of acids was produced by different species. Maximum six acids were produced by *Penicillium citrinum*, while five acids each were found in culture filtrate of of *Acrophialophora fusispora*, *Aspergillus sulphureus*, *Cladosporium cladosporoides*, *Penicillium fellutanum* and *P. simplicissimum*. The results clearly indicate that there was no definite correlation between the number of total acids produced by a fungus and titrable quantity of these acids as evidenced by *P. fellutanum* which showed maximum acidity 8.65 and only five acids. On the other hand, *P. citrimum* produced maximum number of acids but only 5.05 acidity.

These variations may be attributed to the discrepancies in the capacity of utilization of carbohydrates by fungi. Usually, the aliphatic acids (volatile and non-volatile) are produced by the enzymatic breakdown of carbohydrates (Cochrane 1958). The main schemes for this anaerobic dissimilation are Embden Mayerhof Parnas (EMP) and Pentose Phosphate (HMP) cycles. The HMP scheme differs from glycolysis (EMP) in that aldehyde carbon of sugar is liberated as CO_2. The end products of these schemes undergo tricarboxylic and other related cycles to produce these organic acids.

All the fungi do not have identical pathways for these activities. Therefore, several types of metabolites are excreted by a particular species (Rainbow & Rose 1963) and these substances may be different in different cases. The microorganisms are well known for their variability and phenomenon of physiological and biochemical adjustment in relation to environment. Under changeable microecological and microclimatic conditions, fungi undergo adaptation which influence the pattern of their physiological cycles of metabolisms. These microecological conditions may be types of soil aeration, available oxygen, CO_2, carbon, nitrogen, phosphorus, sulphur and other trace elements, *p*H, temperature and moisture of soil which exert direct or indirect influence in the physiological and biochemical adaptation of these fungi in soil (Ainsworth & Sussman 1968). Influence of these individual factors has also been confirmed during fermentation process of various organic acids by several workers; viz. Ward et al. (1936) for lactic acid, Hesseltine (1970) for kojic acid, Herric et al. (1928) for gluconic acid, Kinoshita et al. (1929) for itaconic acid. Similarly, Prescott and Dunn (1959), Restrick (1949) and Walker (1949) have reviewed voluminous literature on the subject. On the basis of the present study only four fast acid producing fungi have been selected for the investigation of various aspect for their use in large scale production of these acids. Further work on this aspect is in progress in this laboratory.

Acknowledgements

Authors are thankful to the Principal, Agra College, Agra for facilities and Director, CMI, Kew, England for identification of fungal cultures.

References

Ainsworth G C and Sussman A S 1968 *The Fungal Population* (New York & London: Academic Press)

Brown W G 1939 Micro-separations by chromatographic adsorption on blotting paper; *Nature* **143** 377–388

Casida L E 1964 Industrial Microbiology (New York: John Wiley & Sons)

Cochrane V W 1958 *Physiology of Fungi* (London: Chapman & Hall)

Foster J W 1949 *Chemical Activities of Fungi* (New York: Academic Press)

Hargreaves C A 1956 Quoted as personal communication; in *Techniques of Radiobiochemistry* ed. S Arnoff (Iowa: Iowa State Coll. Press, Amer)

Herric H T and May O E 1928 The production of gluconic acid by *Penicillium luteum-purpurogenum* group. II. Some optimal conditions for acid formation; *J. Biol. Chem.* **77** 185–195

Hesseltine C W Sorenson W G and Smith M 1970 Taxonomic studies of the aflatoxin producing strains in the *Aspergillus flavus* group; *Mycologia* **62** 123

Kinoshita K 1929 Formation of itaconic acid & mannitol by a new filamentous fungs; *J. Chem. Soc. Japan* **50** 583–593

Lugg J W H and Overall B T 1947 *Austral. J. Sc. Res. Ser.* **A1** 98 (Cited from Malo lactic acid fermentation)

——, —— 1967 *Adv. Appl. Microbiol.*; **9** 235–279

Martin A 1961 *Introduction to Soil Microbiology* (New York)

Prescott S C and Dunn C G 1959 *Industrial Microbiology* (New York)

Rainbow C and Rose A H 1963 *Biochemistry of Industrial Micro-organisms* (New York)

Raistrick H 1949 A region of biosynthesis (Bakerian Lecture); *Proc. Roy. Soc.* **A199** 141–168

Sharma K D and Mishra K K 1977 Soil fungi of Banshipaharpur (Rajasthan); *Proc. Indian natn. Sci. Acad.* **B43** 55–56

Waksman S A 1952 *Soil Microbiology* (New York)

Ward G E, Lockwood L B, May O E and Herric H T 1936 Studies in the genus Rhizopus. I. The production of dextrolactic acid; *J. Amer. Chem. Soc.* **58** 1286

Walker T K 1949 *Advances in Enzymology* **9** 537