C H A P T E R - III

MATERIALS AND METHODS

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(I) Isolation of fungi:

Soil samples were collected from diverse places in and around Raipur. The fungi from these samples were isolated according to the standard methods (Waksman, 1932; Warcup, 1960; Durbin, 1961; and Garret, 1963). Pure cultures of the fungi were obtained by repeated subculturing. The cultures were stored at low temperature and were used for further studies.

(II) Screening of fungi for organic acid production:

(1) Primary screening:

Preliminary survey of fungi for their initial capacity to produce organic acid(s) was done following the method of Davis (1948). The organisms were grown in peptone-glucose-agar medium (pH 5.5) containing 0.04% bromo-cresol green indicator. The normal colour of this medium was deep blue green. In case of organic acid production, the zone surrounding the colony changed from an intense blue green to yellow. On the basis of the size of the acid zone and degree of change in the indicator's colour the organisms were classified into weak and strong acid producers.

(ii) Secondary screening:

The fungal organisms that were found as strong producers of organic acid(s) in the preliminary screening were further tested in the broth medium. They were grown in Sabouraud's broth medium consisting of (g/liter) glucose, 40; peptone, 10 and water, 1 liter (pH 4.5). Each organism was grown separately in 40 ml of the above medium in 150 ml Erlenmeyer flask in triplicate. Incubation was done in still state for 7 days in B.O.D. incubator at 28 ± 2°C. The culture filtrates were then analysed for their organic acid content, chromatographically. The organic acid spots on the chromatograms were identified by co-chromatography and their quantities determined colorimetrically.

(III) Assay procedure:

(i) Chromatographic procedure:

(a) Chromatography:

For qualitative and quantitative determination of organic acid content of culture filtrates, the separation of individual organic acids was carried out by ascending paper chromatography (Hulme, 1961). The solvent used was n-butanol : acetic acid : water (4 : 1 : 5, v/v). The solvent was prepared 12 hours in advance of its use. At the time of use, the solvent mixture was poured in a separating funnel, shaken vigorously and the two layers allowed to separate.

The aqueous lower layer was drained out. Only the upper organic layer was used as developing solvent.

For chromatography whatman chromatographic paper No. 1 (40x40 cm) was used as adsorbent. On each sheet of paper, a series of spots were plotted (10 - 20 µl) at points 3 cm from the bottom of the paper and 3 cm apart. At each end of the series, also a mixture of known organic acids and/or control were plotted. Self-filling graduated micropipettes of 10 µl capacity were used for plotting purposes. In order to keep the spot size to a minimum each aliquot was allowed to dry before the next was applied. The spotted papers were then placed in chromatographic chamber for development. After the solvent was run to the required height; the chromatograms were taken out, dried at room temperature and then they were sprayed with aniline-d-xylose reagent. The chromatograms were dried in a hot air oven at 120°C for 30 minutes. The organic acid spots on the chromatograms were identified with the help of the spots of known organic acids run simultaneously, by their Rf values, and their patterns on the chromatograms.

(b) Colorimetry:

For colorimetric determination, after the aniline--d-xylose reagent sensitive spots or organic acid spots

on the chromatograms were developed to their maximum intensity, they were outlined by a lead pencil and then cut and placed in 5 ml of 70% aqueous ethanol for elution. In case of faint spots however, elution was done from 3 replicate spots.

The colorimetric determination of the eluates was done at 525 nm in a Specol-Spectro Colorimeter. Eluates of the "reagent-blank" zones of the chromatograms served as the reference solution. The optical density readings obtained were compared with the standard calibration curve drawn for citric, fumaric or tartaric acid. The quantity of each of the spots was measured from the standard curve as mg/100 ml.

(ii) Gravimetric procedure for citric acid determination:

In case of substantial yields, the quantity of citric acid was also determined gravimetrically (Agnihotri, 1965). To a 50 ml aliquot of the culture filtrate 10 ml of 10% calcium chloride solution was added and neutralized with 10% solution of ammonium hydroxide. Two ml of 20% HCl solution was then added to it. The solution was warmed for a short while and then allowed to cool. Oxalic acid present in the filtrate was precipitated as calcium oxalate which was removed by filtering the solution through Whatman filter paper No. 42. The filtrate and the washings

were combined and concentrated, and, while hot, ammonium hydroxide was added to bring about neutralization. The citric acid of the filtrate got precipitated as its calcium salt. The solution was concentrated to a volume of 25 to 30 ml and filtered through pre-weighed Whatman filter paper No. 42. The precipitate was washed with hot water and then with 50% alcohol. It was then dried at 110°C and the amount of calcium citrate determined. The amount of citric acid was finally determined, multiplying the weight of calcium citrate by the factor 0.7709.

(IV) Estimation of mould growth:

The amount of mould growth was estimated by determining the dry weight of the mycelium formed. At the end of the incubation period, the contents of the culture flask were transferred to a pre-weighed Whatman filter paper No. 1, washed thrice with distilled water and the mycelium finally dried at 60°C for 24 hours before weighing.

(V) Estimation of residual sugar:

Estimation of residual sugar was done with Dreywood's Anthrone reagent (Morris, 1948). To each 2 ml of the culture filtrates, 3 ml of the reagent (0.0?% solution of Anthrone in sulphuric acid) was added and mixed thoroughly by brisk swirling of the

test tube. After the mixture was cooled, the tubes were kept in a water bath for 15 minutes. The blue colour developed was measured at 620 nm in a Specol Spectro Colorimeter against the blank containing only water and the reagent. The optical density readings obtained were compared with the standard caliberation curve drawn for glucose, and the quantity of sugar determined.

(VI) Assay of Acid and Alkaline Phosphatases:

(a) Enzyme preparation:

In each case 1 g mycelium was taken in 10 ml cold N-saline and thoroughly homogenised. The homogenate was made cell free by centrifugation and used directly as enzyme preparation.

(b) Enzyme assay:

It was done according to the method described in 'Hawk's Physiological Chemistry'. Sodium β-glycerophosphate of pH 5.0 and pH 9.7 served as the enzyme substrate for the acid phosphatase and the alkaline phosphatase respectively. In each determination 1 ml enzyme preparation was mixed in 9 ml of the substrate and incubated for 1 hour at 37 $\pm$ 1°C. After the due incubation time the reaction was stopped by adding 2 ml 30% TCA to the reaction mixture, in both the test and control samples. The enzyme activity was measured

in terms of the amount of Pi liberated in each sample. For taking colorimetric readings the reaction mixture was then heated with 1 ml ammonium molybdate solution and 0.4 ml 1,2,4-aminonaphthosulfonic acid to produce colour after reduction of the phosphomolybdic acid, if any, formed from ammonium molybdate with the enzyme liberated Pi, in the reaction mixture. The over all reaction sample was read over 'Specol' at 660 nm. The enzyme activity has been calculated as the amount of Pi(µg) liberated per ml enzyme preparation in one hour from the difference of the Pi liberated in control and test samples, separately for the acid and alkaline phosphatases.

(VII) Isolation and identification of Tartaric acid(TA):

For isolation of TA from the culture filtrate, it was first precipitated in the form of its calcium salt. To 1 litre of the filtrate, therefore, 5 g of $CaCl_2$ was added. The precipitate was then dissolved in 1% sulphuric acid. On heating this solution, $CaSO_4$ got precipitated which was removed by filtering the solution. The clear solution was concentrated on water bath to a volume of 50 to 60 ml and then left for crystallization. Repeating the process of crystallization 5 to 6 times, finally, pure crystals of TA were obtained.

In order to confirm the product as TA, the following tests were performed:

(i) Silver mirror test: To 1 ml solution of the product or culture filtrate a pinch of sodium bicarbonate was added. After it had stopped giving effervescence a drop of concentrated HNO_3 was added to dissolve the excess sodium bicarbonate. Now, an excess of ammonium hydroxide was added and the solution was boiled till there was no further smell of ammonia. One ml of Tollen's reagent was then added through the wall of the tube and the test tube was placed on water bath at 60-70°C for 10 to 15 minutes.

The formation of silver mirror on the walls of the tube was considered as a positive test for TA.

(ii) Resorcinol test: To 1 ml solution of the product, a few crystals of resorcinol were added. The development of a red colour with the addition of 1 ml of concentrated H_2SO_4 in this solution confirmed the presence of TA.

(iii) Fenton's test: (A specific test for TA):

To 1 ml of culture filtrate or the solution of the product, 2 ml concentrated solution of NaOH and 1 ml of hydrogen peroxide were added. On addition of an excess of (approximately 5 ml) freshly prepared ferrous sulphate solution the formation of violet coloured precipitate confirmed the presence of TA.

(iv) Ammonium meta-vanadate test (specific test for TA):

To 1 ml filtrate or the solution of the product, a few drops of acetic acid and 1 ml of 5% ammonium meta-vanadate were added, the development of red colouration, specifically confirmed the presence of TA in the test solution.

The ammonium meta-vanadate solution was used also in the form of spray reagent for chromatography of tartaric acid. When the paper chromatograms of the filtrate (or of the product) were sprayed with this solution, red coloured spots on the chromatograms confirmed the identity of the product as TA.

(v) Melting point test: Finally, the melting point of the product was determined, which was found to be 167°C, against the 169°C of the authentic sample.

(VIII) Experimental Studies:

The capacities of the selected organisms Aspergillus japonicus, Saito (IMI-243984) and Rhizopus oryzae, Went and Geerligs (IMI-244011) for production of citric, and fumaric and tartaric acids, respectively, were investigated under varying cultural conditions. Studies were made to see the effects of basal medium, incubation period, pH, temperature, carbon source, nitrogen source, carbon/nitrogen ratio, phosphorus source, magnesium sulphate concentration, trace elements, and also the low molecular weight alcohols.

To make the processes commercially feasible efforts have also been made to see the possibility of utilizing some crude sources of carbohydrate in place of chemically pure sugars.

Unless otherwise specified, for these experimental studies the cultures were grown in 150 ml culture flasks, containing 25 ml of the respective production medium. The modified medium of Richard (containing all but sucrose, in just half of the original concentration) was used as Citric Acid Production Medium (CAPM); the Cole's tartrate medium (original) as Fumaric Acid Production Medium (FAPM); and the modified Cole's medium (containing ammonium chloride in place of ammonium tartrate and buffered with phosphate:phosphate buffer of pH 6) as Tartaric Acid Production Medium (TAPM).

Inoculation was done with 1 ml of spore suspension containing approximately $5x10^7$ spores suspended in sterile twin-80 (1:10,000). The cultures were incubated at 28 ± 2°C for 7 days in B.O.D. incubator in still state. At the end of the desired period of incubation the cultural broths were filtered and the volume of the filtrates made upto 25 ml. 15 ml of each of these culture filtrates was then frozen immediately for further analysis.

(i) Effect of Basal medium:

To select the best possible type of basal medium for the production of organic acids by selected fungal

organisms (*A. japonicus* and *R. oryzae*) the following 8 types of basal media (Agarwal and Hasija, 1980) were considered initially:

1. Asthana and Hawker's
2. Brown's
3. Cole's tartrate
4. Coon's
5. Czapek's
6. Glucose asparagine
7. Richard's
8. Sabouraud's

Each of the filtrates was then taken to determine (a) final pH (b) amount of residual sugar and (c) organic acid content - qualitative and quantitative.

(iii) Effect of Incubation period:

Studies were made to see the effect of incubation period on the production of organic acids by the selected fungal organisms i.e., CA by *A. japonicus*, and FA and TA by *R. oryzae*. The organisms were grown in their respective best broth media and 3 flasks in each case were harvested daily upto 15 days from the day of the inoculation. The mycelial mats and the culture filtrates were obtained; and the organic acid and residual sugar contents of the filtrates were determined.

(iii) Effect of pH:

To study the effect of hydrogen ion concentrations, the pH of the media before inoculation was adjusted within the range 1-10 , with a difference of pH 1. For the purpose the original pH(s) of the CA, EA and TA production media which were found to be 5.1, 5.6 and 5.4 respectively were brought to the required pH-levels with the help of 0.1 N HCl or NaOH solutions. Incubation was done at 28 ± 2°C, for 9 days in case of CA production and for 7 days in case of EA and TA production. In case of TA production the media also contained 10 ml of phosphate: phosphate buffer of the required pH.

(iv) Effect of Temperature:

In order to ascertain the effect of temperature, the organisms were grown in their respective best media and 3 flasks in each case were incubated at 10, 15, 20, 25, 28, 30, 32, 35, 40 and 45°C. Higher temperatures were not tried because even at 45°C none of the organisms grew well. To avoid the lag effect, the temperatures of the incubators were maintained at least an hour before incubation. The period of incubation and the pH of the media, certainly, were at their optimum levels in every case.

(v) Effect of Carbon sources:

For investigating the effects of various carbon sources, the carbohydrates taken were: glucose, mannose,

galactose and fructose from monosaccharides; sucrose, maltose, lactose and raffinose from disaccharides; and starch and cellulose from polysaccharides. In addition, mannitol - a sugar alcohol was also tried. Thus for the production of CA the original carbon source sucrose of the modified Richard's medium (CAPM), was replaced by equivalent amounts of the different carbon sources under test. The experimental media contained a single carbon source equivalent to 21.09 mg-C-/ml. All other ingradients otherwise remained the same. Similarly for FA and TA production the original C-source glucose of FAPM and TAPM was substituted by the equivalent amount (8 mg-C-/ml) of the different carbon sources. The cultures were incubated for 3 days at 30°C in case of CA production while, for FA and TA production, incubation was done for 7 days at 30°C.

(vi) Effect of Nitrogen sources:

To study the effect of various nitrogen sources, the potassium nitrate and ammonium tartrate of CAPM and FAPM, respectively were substituted by different nitrogen compounds viz., potassium nitrate, sodium nitrate, ammonium nitrate, ammonium sulphate, magnesium nitrate, sodium nitrate, peptone and asparagine. The experimental media contained single nitrogen source equivalent to 0.69 mg-N-/ml in case of CA production while, 1.52 mg-N-/ml in case of FA production. All other

ingredients of the media otherwise were the same. The cultures, after being incubated, were harvested and the culture filtrates analyzed for organic acid and residual sugar contents.

(vii) Effect of C/N-ratios:

The effects of varying levels of carbon and nitrogen concentrations were also investigated. For the purposes, 56 different ratios of sucrose and potassium nitrate (the best source of carbon and that of the nitrogen, respectively) have been tried which can be followed easily from the table 10 depicting the results of C/N-ratios.

(viii) Effect of Magnesium sulphate concentration:

To study the effect of the different concentrations of $MgSO_4$ on the yield of CA and FA the concentration of the former was varied from 10 to 100 mg/100ml with a difference of 10 mg. The other conditions of the culture remained the same as described in the previous studies.

(ix) Effect of the Source and the Concentration of Phosphorus compounds:

Assimilable phosphates are also one of the essentials for the growth and the metabolism of the moulds. To evaluate the effect of different phosphorus

compounds therefore, potassium di-hydrogen phosphate, di-potassium hydrogen phosphate, sodium di-hydrogen phosphate and di-sodium hydrogen phosphate were added singly in CA, cA and TA production media in the amounts so as to furnish 717, 286 and 286 mg of phosphorus/litre.

Also the effect of different concentrations of KH_2PO_4 (the best source o. phosphorus) was studied. The different concentrations of KH_2PO_4 tried, in case of CA production were - 100, 200, 250, 300, 400 and 500 mg/100 ml, while that for cA and TA productions were - 25, 50, 75, 100, 125, 150 and 250 mg/100 ml, respectively.

The effect of potassium di-hydrogen phosphate was investigated, also with respect to the growth and the acidogenic phases of citric acid fermentation. For the purpose the culture medium was supplied with (100 mg/100 ml) of KH_2PO_4 in the following manner (a) only during growth phase (from 1-5 days) (b) only during acidogenic phase (from 5-9 days) (c) throughout the fermentation (from 1-9 days) and (d) not at all.

(x) Effect of Trace elements:

To investigate the effects of trace elements on the production of organic acids by the fermenting molds, the trace metals and the concentrations, thereof, considered were as follows:

Trace metals used: Zn, Fe, Mn and Cu.

Concentrations tried for CA production:

i. To see the effect of individual elements:

0.5, 1, 2, 4 and 10 ppm.

ii. To see the effects in combinations of two's, three's and four's:

0.5 ppm of each element.

Concentrations tried for FA and TA production:

i. To see the effect of individual elements:

5 and 15 ppm.

ii. To see the effects in combinations of two's, three's and four's:

5 ppm of each element.

To eliminate the effects if any due to anions, only the sulphates of these metals were chosen for this study.

For the accuracy of the results necessary precautions were duly taken. Only AR grade chemicals (sugar and metallic salts) and Pyrex glass apparatus (the culture flask, funnels and beakers etc.) were used. Throughout the experiment deionised water was used for cleaning purposes while, only triple distilled water was used for preparing salt solutions, HCl and NaOH solutions, media, and inocula.

(xi) Effect of Alcohols:

If added in the medium, the low molecular wt. alcohols are known to increase the production of organic acids by antagonizing the bad effects of trace elements (Moyer, 1952). To find out the suitable-most alcohol (or combination of alcohols) and then also the optimum concentration, thereof, methanol, ethanol and propanol were added in the media separately and in combinations in the amounts so as to make their final concentrations 1, 2, 3 or 4% in case of CaPM or 1, 1.5, 2, 2.5 and 3% in case of FAPM and TAPM. The addition of alcohol, to the cool sterile medium, was done just prior to inoculation.

In case of CA production, the effect of methanol (found as the best alcohol), was studied also against the time of its application. To the medium was added, 1, 2, 3 and 4% of methanol, after 0, 1, 2, 3, 4 and 5 days of the inoculation.

(xii) Utilization of crude Carbohydrate sources:

The possibility of utilizing certain crude sources of carbohydrate for the production of CA, FA and IA was studied. The sucrose from CaPM and glucose from FAPM and TAPM were replaced by equivalent amounts of the crude carbohydrates under test i.e., powders of sweet potato and knol-khol tubers and mango kernel

(oil mill residue). Also with a view to overcoming the problem of trace elements and thereby to obtain increased yields of these organic acids, the media were either treated with $K_4Fe(CN)_6$, Ion exchange basic alumina (of 8, 9 & 10 pH) and EDTA or were supplied with the low molecular wt. alcohols (methenol), ethanol and propanol) (Table - 14). The cultures after being incubated were harvested and the culture filtrates analyzed as before.

(xiii) Mutational studies:

In order to obtain high yielding mutated strains of Aspergillus japonicus (IMI-243984), the parent culture was exposed to the mutagen ultra-violet rays. For the purpose the organism was grown on the slants of Richard's agar for 10 days. Scraping the spores, a suspension was prepared in sterile tween-80 solution (1:10,000). The preparation was thoroughly shaken and filtered twice through sterile cotton to remove spore clumps. The concentration of the spore suspension was finally made upto 10-12 spores/ml. The samples were irradiated with ultra-violet rays (240 V, 300 W) for 10, 15 and 20 minutes from a distance of nine inches (Gadkar and Vyas, 1970). After irradiation, the suspension was spread on Richard's agar medium containing bromo-cresol green indicator.

From among the colonies produced by a very few surviving spores, 7 colonies, apparently distinct and also giving deep yellow acid zones, were selected. These were then tested for their CA production capacities in Richard's synthetic medium, as well as in the medium employing crude sources of carbohydrates.