‘Albino’ mutation in rice bacterial blight pathogen

The cultures of *Xanthomonas oryzae* pv. *oryzae*, the pathogen of bacterial blight of rice, depicted a characteristic yellow-pigmented growth on conventional culture media viz., Nutrient Agar (NA), Peptone Sucrose Agar (PSA) and Yeast Glucose Chalk Agar (YGCA). The pigmentations, chromatic intensity of which varied from light yellow to deeper yellow among the isolates, was a universal xanthomonadic character of the bacterium. As a result of mutation, non-pigmented ‘albino’ strains were, however, encountered in some xanthomonads.1,2 Natural occurrence of such episodes have not been recorded in rice bacterial blight pathogen so far.

In 1991, for studying colony characters of the bacterium, suitable dilutions of actively-growing young cultures were spread on PSA medium in Petri plates and incubated at 28°C (±) for 5 days. Characteristically yellow colonies appeared in all the plates, but a non-pigmented (white) xanthomonad-like colony was, however, detected among population of yellow colonies in one plate. The white as well as yellow colonies were transferred to sterile water columns for purification. Both colonies yielded pure population of respective colony types. Stability of chromatic character was confirmed by culturing white and yellow forms on NA, PSA, YGCA media at 20, 25, 30, 35°C. Virulence of single colony cultures of white and yellow forms was tested in plants by leaf-tip clipping inoculation. The white cultures were equally pathogenic as the yellow ones inducing identical leaf blight lesions. Reisolations yielded pure population of respective forms.

The pigmented and non-pigmented cultures revealed similarity in cultural, morphological and physiological characters. Chromatically distinct forms were culturally smooth, mucoid, raised, glistering, butyrous, opaque and spreading; bacterial cells were rod-shaped, Gram negative, encapsulated, non-sporing and possessed single polar flagellum. Cultures of both categories were strictly aerobic; utilized glucose oxidatively; hydrolysed gelatin but not casein and starch; produced ammonia, hydrogen sulphide and catalase but not urease, oxidase peroxidase, arginine hydrolyase and asparaginase; nitrate reduction, indole production, methyl-red and Voges-Proskauer tests were negative; asparagin was not utilized as a sole source of C and N; no acid production in litmus milk, neither peptonization nor curdling; produced acid without gas from glucose, fructose, mannose, xyllose, cellobiose and sucrose (slight) but not from arabinose, ribose, rhamnose sorbose, lactose, maltose, cellulose, starch, dextrin, insulin, glycogen and sugar alcohols like sorbitol, mannotol, dulcitol; readily utilized organic acids of citric acid cycle but failed to utilize oxalic, tartaric and benzoic acids.

The non-pigmented culture was identified to be an ‘albino’ mutant strain of yellow-pigmented *X. o. pv. oryzae*, which did not reveal variation from original chromatic character in either virulence or xanthomonadic bacteriological characters.

We further evaluated in vivo interaction between ‘albino’ (W) and chromatic (Y) cultures. Aqueous bacterial suspensions (10^7 cfu/ml), containing both cultures at 3 concentration ratios (W0:1:9:Y, W5:5Y, W9:0:1Y), were inoculated in TN1 plants and isolations were attempted from inoculated leaves at a distance of 10 cm from inoculated sites after 10 days. The isolated population depicted the following colony patterns in all cases of concentration ratios: W > Y, W = Y, Y > W, thus, suggesting absence of any pathological interaction between the chromatically distinct cultures.

Pathological behaviour on 5 vars. (BJ1, DV85, IR1545-339, Tkm6, TN1) also did not reveal noticeable variation between white and yellow forms (IR1545-339, TN1 as susceptible and BJ1, DV85 Tkm6 as resistant to both cultures).

The investigation provided substantial evidence to conclude that chromaticity and virulence in xanthomonads were genetically independent characters. Our observations, thus, could not support the suggestion from the Philippines (a personal communication from IRRI, Manila) that ‘albinosis’ resulted in development of new race in *X. o. pv. oryzae*. It is relevant to point out that Philippines’ ‘albino’ strain was obtained by using mutagenic agent and possibly genes of chromaticity as well as virulence were involved in the mutation resulting in development of a new ‘albino’ (non-pigmented) race.


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Strain improvement of hydrocarbonoclastic bacterial isolates from mangals of Andaman

Any oil spill pollution is potentially dangerous and of major environmental concern. Out of at least 157 spills in the tropics between 1974-1990, 99 were close to reefs and mangroves. Environment within the mangroves and reefs is sheltered and rarely flushed by pounding waves resulting in entrapment of oil around the lower trunks and breathing roots, thus exerting toxic effects and are made worse by the lingering presence of oil trapped in the sediments. Removal of such pollutants from the affected ecosystem is primarily carried out by indigenous hydrocarbonoclastic microorganisms. The rate at which this removal takes place is limited by environmental conditions, mainly abiotic factors which influence the rate of growth and enzymatic activities of these microbes and/or by a lack of adapted microbial population. Accidental or willful marine oil spill could not be stopped till date and is definitely a difficult proposition. The latest incidents of oil spill were along the Louisiana coast and southern coast of Yemen covering ~25-30 km in July 1995. Recent developments in biotechnology and microbial technology may be promising to meet the challenge. Action plan for any oil spill event is depicted in Figure 1. Seeding with oil-eating microorganisms and augmenta
tion of microbial processes by fertilizer application are the two approaches considered for bioremediation of marine oil spills. Genetically engineered hydrocarbon degraders have been developed, but none of them has actually been applied to an oil spill partially because of their inability to degrade all the hydrocarbon pollutants and also because of government-imposed restrictions to the deliberate release of genetically engineered microorganisms. Recombination of gene pool in nature is well established but the debate on manmade recombination by genetic engineering still continues and the integrity, stability and fate of such recombinants in nature stands challenged. This scenario necessitates the improvement of the strain of potential naturally occurring oil-eating microbes to enhance toxic petroleum hydrocarbon degrading efficiency in order to maintain a sound coastal, marine and mangrove ecosystem.

While studying the special physiology of microbial flora associated with mangals of Andaman close to major shipping corridors, we could get a number of hydrocarbonoclastic bacteria. In an effort to enhance the efficiency of such bacterial isolates, we tried to adapt a few potential isolates with different hydrocarbon sources since our earlier report. After thorough screening, three mutants, originating from our earlier five hydrocarbon degrading isolates (when put to rigorous selection pressure in directed way) were studied in detail in pure form and in combination (mixed form). We found dramatic improvement in *in vitro* petroleum hydrocarbon degradation with engine oil, gear oil, 2T mobil oil, xylene, toluene and crude oil at the rate of 2% (v/v) in minimal salt medium. The initially clear medium became turbid because of bacterial growth with or without obvious emulsification of oil (Figure 2, Table 1). Seventy two hours time was enough to emulsify and utilize more than 90% of oil by all the three mutants (belonging to two genera) as evidenced by carbon tetrachloride extraction of residual oil. However, the mutants and the other five isolates utilized xylene and toluene in almost equal efficiency as studied by UV spectrum analysis (Figure 3). UV spectra of xylene and toluene utilization were taken with appropriate reference (1% substrate in MSM) and only MSM for base line correction.

![Figure 1. Schematic representation of oil spill pollution control strategy.](image1)

![Figure 2. Utilization of different petroleum products by mutants. a. Gear oil; b. 2T Mobil oil; c. Engine oil; d. Crude oil. RC: Reference control, 1; 12/1-OS/Cp; 2; 17/1-Ot/Cp.](image2)

<p>| Table 1. Growth of hydrocarbon degrading mutants* on petroleum products |
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<table>
<thead>
<tr>
<th>Isolates</th>
<th>Gear oil</th>
<th>Engine oil</th>
<th>2T Mobil oil</th>
<th>Crude oil</th>
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<tr>
<td>12/1-OS/Cp</td>
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<td>17/1-Ot/Cp</td>
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*Mutants were grown for 72 h in mineral salt medium containing 2% (v/v) of the substrate. + sign indicates 1 to 4 scale of hydrocarbon utilization.
UV spectrum of xylene showed a minor peak at 240-280 nm which was completely absent for all the eight improved isolates showing visible growth, whereas in case of toluene one minor peak at 228-280 nm showing subpeaks at 262 nm and 270 nm also vanished with almost equal potency. In addition, the major peak in both the cases shifted to the left, indicating utilization of these substrates by the organisms. Substrate-specific stimulation of the isolates in natural and artificial nutritional stressed conditions resulted in the development of these mutants. Interestingly, the mutants did not change the major physiological parameters of the parent strains such as halophilism and mesophilism.

The function of bioemulsifier/biosurfactants is related to the hydrocarbon uptake and is therefore, synthesized predominantly by hydrocarbon-degrading microorganisms. In our experiment, emulsification factor was extracted from culture supernatant by chilled acetone and we found that the factors produced in response to different hydrocarbon sources are not chemically same (Table 2). The response observed in different solvent system, temperature, pH, ninhydrin reagent and anthrone reagent indicated that they were peptidolipid with varying chain lengths. These factors were not produced when the same organisms were allowed to grow in MSM with glucose, sucrose and maltose as carbon source. Several types of emulsifiers have been isolated and characterized. These include glycolipids, fatty alcohols, fatty acids, polysaccharides, glycoproteins, peptides and peptidolipids.

Presence of cryptic genes in the original isolates and the mutants resulting out of environmentally-directed or adaptive mutation is the possible explanation for the improvement of these strains, which is in agreement with earlier studies. This improvement is directly correlated to the enhanced production (2.5-3.1 g l⁻¹) of emulsification factor by the mutants in comparison to the parent isolates (0.4-0.65 g l⁻¹). Thus it appears that these directed mutants (Cpr-dir⁻) need to be maintained and monitored for stability and inheritance pattern in greater detail in presence of different hydrocarbons, pure and mixed form, so that a stable and efficient genotypic consortium can be developed to meet the threat to mangrove and marine ecosystem by any

| Table 2. Characteristics of emulsifying factors produced by hydrocarbon degrading strains. |
|-----------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Colour | White | White | Whitish |
| Consistency | Jelly like | Jelly like | Jelly like |
| Solubility in seawater | 12/I-OS/Cpr²⁺ | 12/I-OS/Cpr²⁻ | 17/I-OW/Cpr²⁻ |
| pH | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 |
| MSM | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Concentration | Partial | Partial | Partial | 60°C 30 min | 50°C 1 h in vacuum | 28°C 1 h in vacuum | Petroleum products | Glucose | Sucrose | Maltose | Petrolcreek reagent | Anthrone/Oxineol reagent | Iodine reagent | Benedict’s reagent | Partial | Partial | Partial |
| Activity after heating | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Productivity with C source | +++ | +++ | +++ | 200 | 250 | 300 | 0.0 | 1.0 | 2.0 | Wavelength (nm) | a | 1 | 1 | 1 |

Figure 3a. UV spectrum of xylene utilized by eight strains of hydrocarbon-degrading bacteria (see text). 1 is the spectrum of xylene in MSM (1% v/v).
eventual oil spillage. Further studies in this direction are in progress.


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A low-cost culture vessel for in vitro culture of plants

The use of tissue culture in crop improvement, afforestation and micropropagation in the ornamental horticulture industry is well documented. One of the major difficulties in expanding propagation techniques to other crop areas is the production cost. Indeed, many tissue culture results are interesting from an academic standpoint, but they are not of much benefit to industries because of the high economic cost. Non-availability of glassware restricts the research progress in developing countries. Mass production of photoautotrophic organisms was successfully carried out by using poly-bag bottles. We report here the proposed design of poly-bag bottle aimed at cutting the cost of in vitro propagation of plants.

Polypropylene bag of dimensions (26.0 x 16.5 cm) was brought from the local market. A collar (dia 3.0 cm height 2.5 cm) was made by cutting a piece from a cylindrical tube of polyvinyl chloride. The polypropylene bag was inserted into the collar. Then the cotton plug was inserted into the mouth of the poly-bag bottle after inoculation of media. For convenience, the mouth of the poly-bag bottle can be widened by changing the diameter of the collar.

Young tobacco (Nicotiana tabacum L.) leaves were cut into small pieces (0.4 cm²), surface sterilized with 0.1% sodium hypochloride and 50% alcohol, washed in sterile water three times and used as explants for callus initiation. The explants were transferred to poly-bag bottle and conical flasks containing 30–40 ml of MS medium supplemented with 2% sucrose, 1 mg/l 2,4-D, 0.2 mg/l BAP and solidified with 0.7% agar. Callus was grown 5 days in dark at 25°C, RH 50–60% and then with 12 h photo-periods. Light intensity was 37 µE m⁻² s⁻¹.

Dissected embryos (half) of green gram (Phaseolus radiates L.), corn (Sorghum vulgare L.) and citrus (Citrus indica L.) were sterilized with 0.1% hypo chloride and 50% alcohol for a few seconds and washed three times in sterile water. Poly-bag bottles were steam sterilized with 30–40 ml of culture medium containing MS salts for 20 min. Sterile embryos were placed on the medium and incubated at 25 ± 1°C under fluorescent light with 12 h photo periods. Callus and seedlings were also raised in 500 ml conical flasks for comparison.

Plant tissue culture is used to achieve many different objectives which require in common the growth of microbe-free plant material in an aseptic environment. Callus culture of tobacco was successfully carried out with poly-bag bottle (Figure 1 a). Several manipulated skills may be applied to get expected results in various aspects of tissue culture with poly-bag bottle and the latter can be used for mass propagation in plant tissue culture industry with low labour cost input. As shown in the photograph (Figure 1 b), seedlings can grow without any fungal/bacterial contamination in poly-bag bottles. The advantages of poly-bag bottles over glassware in in vitro culture of plants are listed in Table 1.