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# Effect of addition of nitrogen, phosphorus and potassium fertilizers on biodegradation of crude oil by marine bacteria

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Role of marine bacteria *viz. Marinococcus albus, Methylobacterium mesophilicum* and *Nocardia otitidiscaviarum* in bioremediation of crude oil contaminated Alang sea coast has been evaluated in the present study. Optimum concentrations of inorganic nutrients N, P & K as urea, super phos and potash fertilizers respectively on growth and degradation has been determined. Addition of optimum concentrations of N (1%), P (0.5%) and K (0.01%) in combination significantly enhanced biodegradation by 22 to 32%. Bioremediation studies indicated that a Bacterial consortium (BC) exhibited maximum growth and degradation on addition of optimum concentrations of N, P and K. Maximum effect in increase in growth was on a BC (0.54 mg/ml) whereas, maximum effect on increase in degradation was on *N. otitidiscaviarum* (31.9%). However, complete degradation was not possible due to the presence of recalcitrant molecules and accumulation of toxic metabolites during biodegradation. Three isolates can successfully be used to formulate a successful bioremediation strategy for oil contaminated marine environment.

[**Key words:** Bioremediation, Biodegradation, Marine environment, Crude oil, N, P and K fertilizers]

## Introduction

Oil entering into marine environment whether from seeps or from anthropogenic sources, is subject to a variety of natural processes and the majority of any oil is removed by biodegradation[1]. The recognition of oil as a complex but largely biodegradable mixture of hydrocarbons and the presence of hydrocarbon degraders in oil-polluted environments, have contributed greatly to bioremediation strategies. Bioremediation is considered to be an efficient, economic and versatile option to treat oil-contaminated sites[2].

Microbial activity in marine environment is generally considered to be limited by low levels of inorganic nutrients as N, P and K that are essential for microorganisms growing on hydrocarbons. Hence, biodegradation of oil stranded marine environment is typically limited by their supply. The application of inorganic nutrients as N, P and K to partially alleviate this limitation has proven to be an environmentally-acceptable and cost effective way to stimulating biodegradation i.e., bioremediation[3,4].

Due to complexity of oil products, biodegradation caused by mixed culture is more effective than that caused by pure culture[5]. This may be due to a broader enzymatic capability that can be achieved and the possible formation of toxic intermediates that can be counteracted by co-metabolic processes[6].

The present study consists of the i) effect of addition of N, P and K fertilizers on growth and degradation of crude oil by *Marinococcus albus, Methylobacterium mesophilicum* and *Nocardia otitidiscaviarum*
ii) bioremediation studies with the above three individual isolates and a bacterial consortium (BC) comprising a combination of the three isolates with addition of optimum concentrations of N, P and K.

## Materials and Methods

***Organisms*** — Three crude oil degrading isolates *viz. Marinococcus albus*, *Methylobacterium mesophilicum* and *Nocardia otitidiscaviarum* were used for this study. These bacteria were isolated from crude oil contaminated sea-water and sediments collected from Alang sea coast, Bhavnagar, Gujarat, known for its extensive ship-breaking yard activities. The isolates were maintained on Zobell Marine Agar[7] and stored at 4ºC until use.

*Effect of addition of Nitrogen (N), Phosphorus (P) and Potassium (K) on biodegradation of crude oil and determination of its optimum concentration* - $10^8$ cells/ml of the above three isolates were inoculated in

Bushnell & Haas Medium (BHM)[8] containing 5 g/*L* of crude oil (determined earlier, data not shown). Media were supplemented each with various concentrations of N (0.1, 0.5 and 1.0%) as urea, P (0.1, 0.5 and 1.0%) as super phos and K (0.01, 0.1 and 1.0%) as potash fertilizers to observe their effect on growth and degradation of crude oil. 50 ml media were dispensed in 250 ml conical flasks. The flasks were incubated at their optimum temperatures for growth and degradation as determined earlier i.e., 30ºC for *M. albus* and 25ºC for *M. mesophilicum* and *N. otitidiscaviarum* (data not shown) on a rotary shaker at 150 rpm. Growth (whole cell protein – mg/ml) and degradation rates (spectrofluorometric analysis - %) were examined from 4$^{th}$ day onwards, at 6 days interval, upto 40 days as described by Vyas & Dave[9]. The controls comprised of inoculated BHM without addition of N, P and K and uninoculated BHM with addition of N, P and K to observe for loss of crude oil due to weathering. The actual degradation rates were calculated by considering the loss of crude oil due to weathering as one of the control. The experiments were conducted in triplicates. The data were subjected to two-way ANOVA. Post Hoc Dunnett's test was performed which compares sets of treatments (various concentrations of N, P and K) against a single control mean using SPSS 13.0.

*Bioremediation studies* — $10^8$ cells/ml of the three isolates and a BC comprising a combination of the three isolates in ratio of 1: 1: 1 were inoculated in BHM supplemented with a combination of optimum concentrations of N, P and K as obtained above. The flasks were incubated at their optimum growth temperatures as mentioned above, while the optimum conditions for a BC was 25ºC and pH 7. Growth and degradation rates were examined from 4$^{th}$ day onwards, at 6 days interval upto 40 days. The controls comprised of inoculated BHM without addition of N, P and K and uninoculated BHM with addition of N, P and K to observe for loss of crude oil due to weathering. The experiments were performed in triplicates. The data have been statistically analyzed by Paired T-test to determine the level of significance using SPSS 13.0.

## Results

The effect of addition of nitrogen on growth and degradation at concentrations 0.1, 0.5 and 1% on *M. albus*, *M. mesophilicum* and *N. otitidiscaviarum* is as shown in Fig I. The Figures clearly indicate increase in N concentration from 0.1 – 1% resulted in increased growth and degradation with maximum growth and degradation at 1% N concentration by all the three isolates. Amongst the three isolates, maximum growth and degradation was exhibited by *N. otitidiscaviarum* (G – 0.79 mg/ml, D – 31%) on 28$^{th}$ day, followed by *M. albus* (G – 0.77 mg/ml, D – 29.3%) on 34$^{th}$ day and *M. mesophilicum* (G – 0.69 mg/ml, D – 22.6%) on 28$^{th}$ day. Addition of 1% N resulted in increase in growth by 0.12 mg/ml and degradation by 6.3% in *M. albus*, 0.08 mg/ml and 5.6% in *M. mesophilicum* and 0.19 mg/ml and 11.9% in *N. otitidiscaviarum*. Thus, maximum effect of addition of 1% N was on *N. otitidiscaviarum* followed by *M. albus*, least was in *M. mesophilicum*.

The effect of addition of P on growth and degradation at concentrations 0.1, 0.5 and 1% by *M. albus*, *M. mesophilicum* and *N. otitidiscaviarum* is as shown as Fig 2. The figures indicate that increase in concentration from 0.1 – 0.5%, resulted in increased growth and degradation by all the isolates, with maximum growth and degradation at 0.5% P concentration. A further addition of 1% had no remarkable effect on growth and degradation. Amongst three isolates maximum growth and degradation was exhibited by *M. albus* (G - 0.71 mg/ml, D - 26.2%) on 34$^{th}$ day, followed by *N. otitidiscaviarum* (G - 0.70 mg/ml, D – 24.1%) and *M. mesophilicum* (G - 0.70 mg/ml, D – 22.2%) on 28$^{th}$ day. Even though maximum growth and degradation was exhibited by *M. albus*, maximum effect in increase in growth and degradation was by *M. mesophilicum* (G – 0.09 mg/ml, D – 5.3%) followed by *N. otitidiscaviarum* and least was on *M. albus* (G – 0.06 mg/ml, D – 3.3%).

Effect of addition of K at concentrations 0.01, 0.1 and 1% on growth and degradation are depicted in Fig.3. . Maximum growth and degradation exhibited by all the three isolates was at 0.01% K concentration. Amongst the three isolates, maximum growth and degradation has been achieved by *M. albus* (G – 0.70 mg/ml, D – 25%) on 34$^{th}$ day, followed by followed by *M. mesophilicum* and *N. otitidiscaviarum* with almost identical growth and degradation (G – 0.66, 0.64 mg/ml, D – 21%) on 28$^{th}$ day. Whereas, the effect of increase in growth and degradation at 0.01% was maximum in *M. mesophilicum* (G – 0.06 mg/ml, D – 2.4%), followed by *M. albus* (G – 0.05 mg/ml, D – 3%) and *N. otitidiscaviarum* (G – 0.06 mg/ml, D – 4.1%). The addition of 0.1% K fertilizer had no remarkable effect

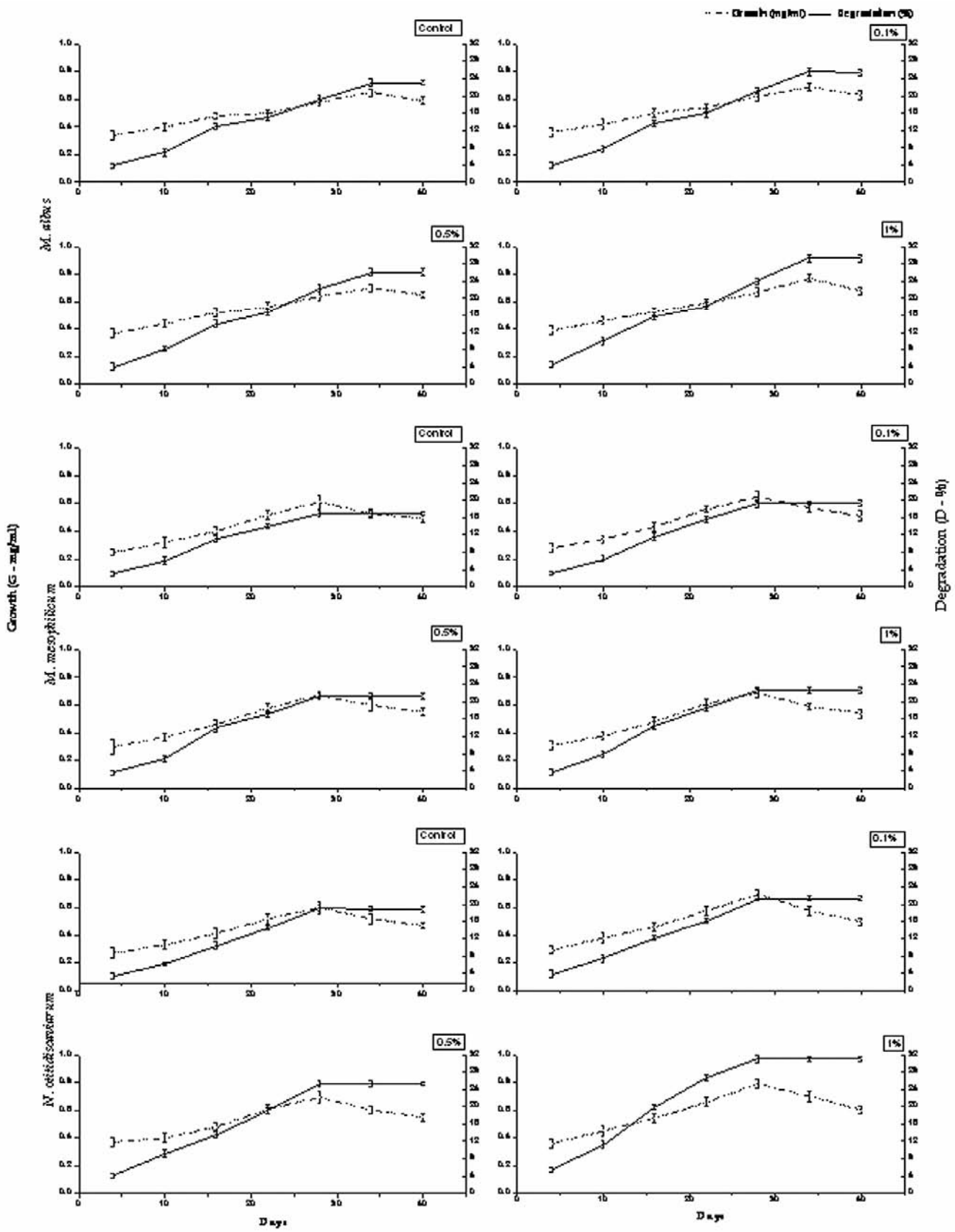


Fig. 1—Effect of addition of various concentration of N (0.1 - 1%) on growth and degradation by the isolates

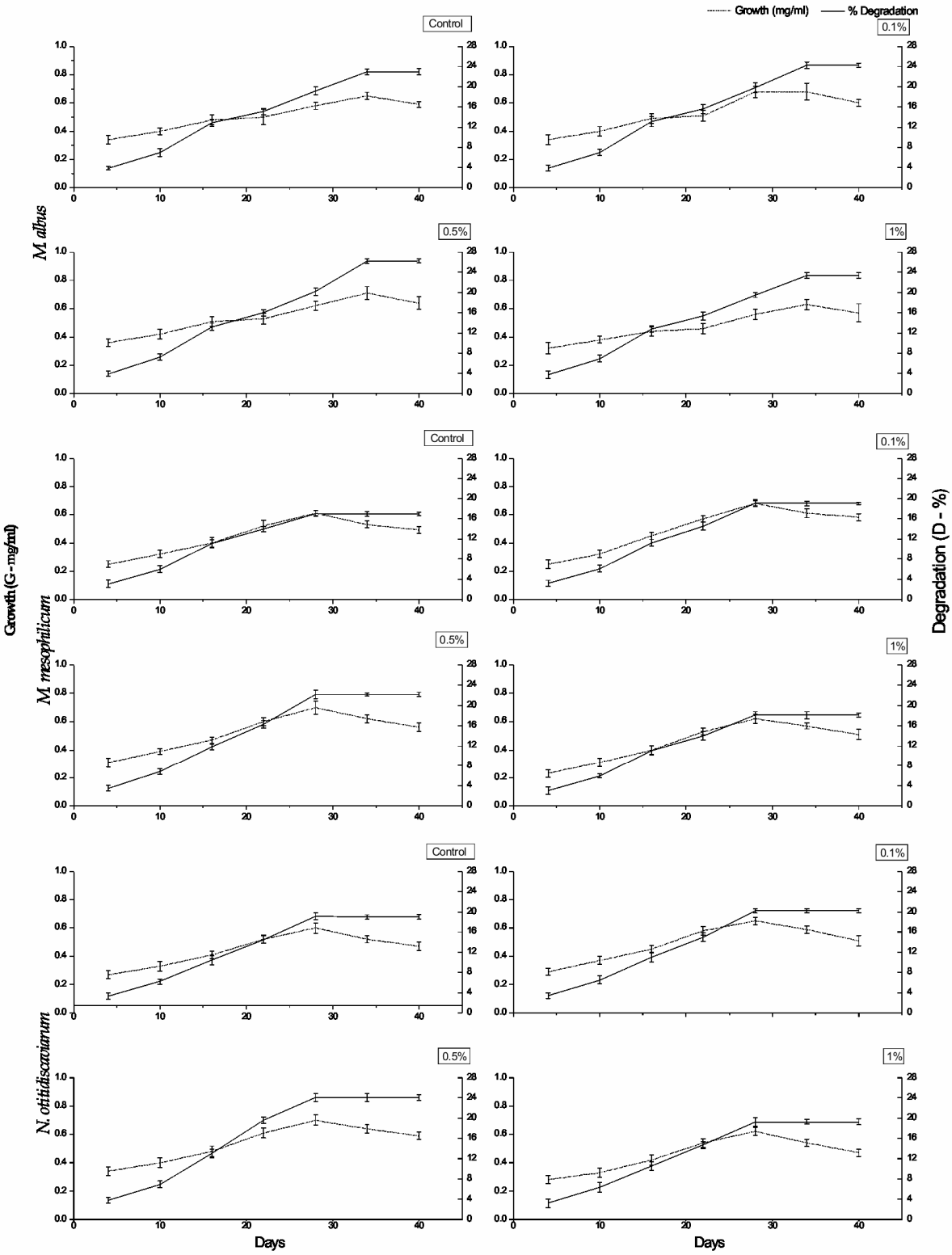


Fig. 2—Effect of addition of various concentration of P (0.1 - 1%) on growth and degradation by the isolates

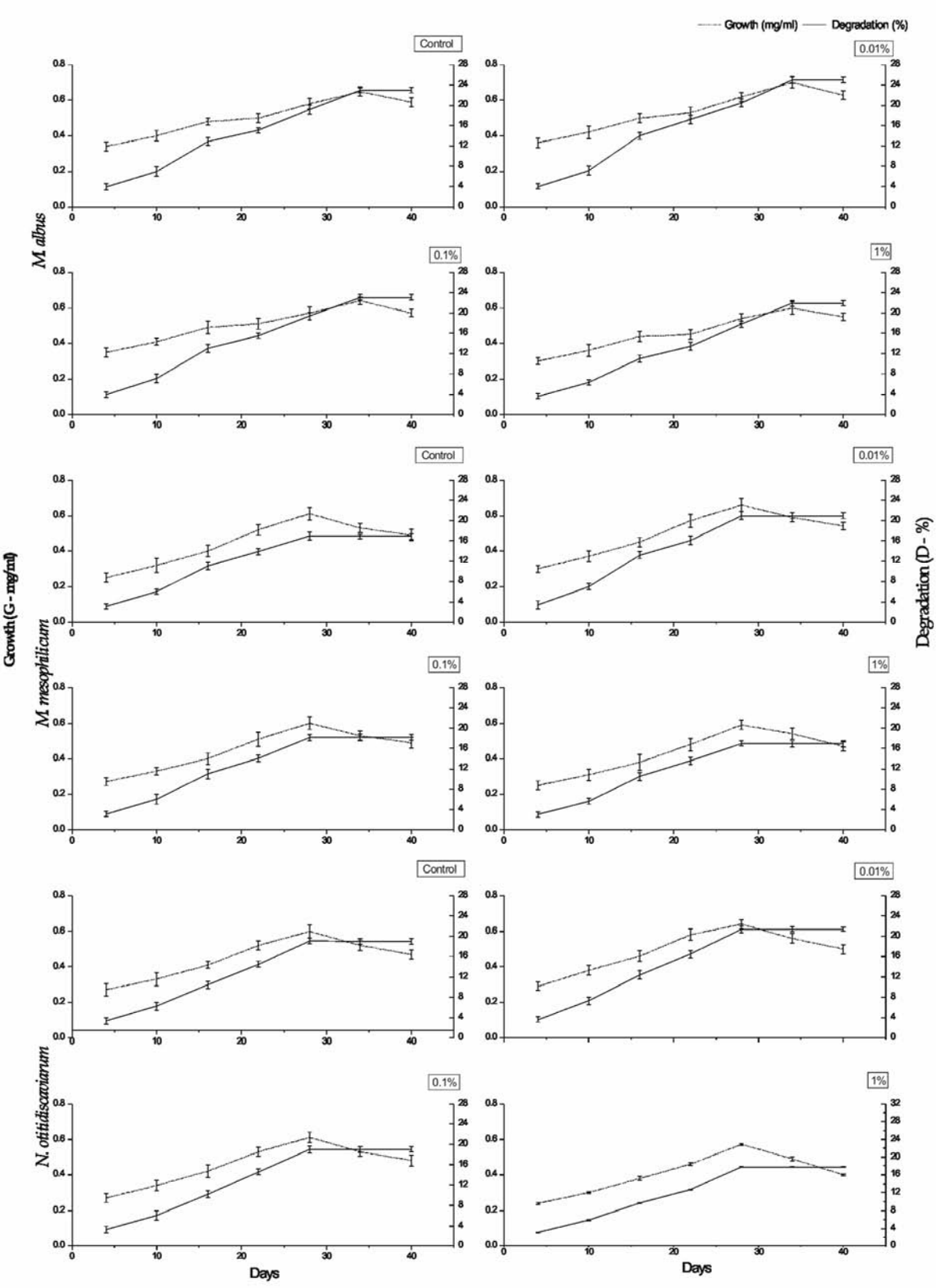


Fig. 3—Effect of addition of various concentration of K (0.01 - 1%) on growth and degradation by the isolates

on both growth and degradation. However, 1% proved to be toxic. It is observed that 1% N, 0.5% P and 0.01% K are optimum for both growth and degradation. With all the variable concentrations of N, P and K, growth declined after reaching its maxima. Whereas, degradation remained stable. However, the organisms exhibiting maximum effect on growth and degradation varied with the fertilizer. It was *N. otitidiscaviarum* on addition of N and *M. mesophilicum* on addition of P and K. Nevertheless, nutrients also had a varied effect. N fertilizer had a maximum effect on growth and degradation followed by P and K.

Two-way ANOVA for effect of addition of N, P and K indicated that the results of growth and degradation are highly significant at $P \leq 0.001$ (Table I). Post Hoc Dunnett's test indicated that the data of effect of addition of N on growth and degradation was highly significant at all concentrations ($P \leq 0.001$) whereas, the effect of addition of P was highly significant at 0.05% concentration ($P \leq 0.001$) and non significant at 1% (Table II). With various concentration of K, the data were highly significant at the least concentration i.e, 0.01% whereas, with higher concentration i.e., 0.1 and 1%, the results were non significant (Table II)

Bioremediation studies with optimized conditions of crude oil concentration (5 g/L), temperatures of 25 and 30 °C and pH 7 have been reported earlier[9]. The results of combined effects of addition of optimum concentrations of N, P and K *on M. albus, M. mesophilicum, N. otitidiscaviarum* and a bacterial consortium (BC) have been presented in Fig 4. *M. albus* showed increase in growth by 0.44 mg/ml and a corresponding two-fold increase in degradation (49.1%) as compared to control. *M. mesophilicum* exhibited increase in growth by 0.28 mg/ml and a corresponding two-fold increase in degradation (40%). *N. otitidiscaviarum* exhibited increase in growth by 0.51 mg/ml and a corresponding three-fold increase in degradation (51.4%). A BC represented maximum increase in growth i.e., 0.54 mg/ml and a corresponding two-fold increase in degradation (60.4%). Thus, though maximum growth and

Table I—Two-way ANOVA of effect of addition of N, P and K on growth and degradation by *M. albus, M. mesophilicum* and *N. otitidiscaviarum*. (Values represent the F value)

| Isolates→ Fertilizer ↓ | Two-way | *M. albus* | | *M. mesophilicum* | | *N. otitidiscaviarum* | |
|---|---|---|---|---|---|---|---|
| | | G | D | G | D | G | D |
| N | Days | 196.88*** | 1443.86*** | 184.97*** | 1237.0*** | 180.6*** | 1940.29*** |
| | Concentration | 26.73*** | 92.20*** | 18.60*** | 147.29*** | 63.87*** | 829.23*** |
| P | Days | 125.16*** | 1727.95*** | 293.75*** | 1318.36*** | 204.74*** | 1260.03*** |
| | Concentration | 11.06*** | 13.58*** | 34.30*** | 82.77*** | 37.52*** | 117.27*** |
| K | Days | 191.19*** | 1485.07*** | 207.95*** | 1020.58*** | 253.83*** | 1647.24*** |
| | Concentration | 24.60*** | 50.36*** | 17.62*** | 83.15*** | 58.63*** | 116.91*** |

* - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$

Table II—Post Hoc Dunnett's test of growth and degradation by *M. albus, M. mesophilicum* and *N. otitidiscaviarum*. (Values represent the mean difference)

| Isolates→ Fertilizer ↓ | Concentration (%) | *M. albus* | | *M. mesophilicum* | | *N. otitidiscaviarum* | |
|---|---|---|---|---|---|---|---|
| | | G | D | G | D | G | D |
| N | 0.1 | 0.03729*** | 1.3905*** | 0.03219** | 1.3838*** | 0.04662*** | 1.7857*** |
| | 0.5 | 0.056*** | 1.9952*** | 0.06057*** | 2.9938*** | 0.07895*** | 4.4071*** |
| | 1 | 0.07905*** | 3.9238*** | 0.07133*** | 4.0457*** | 0.1367*** | 9.30*** |
| P | 0.1 | 0.0295* | 0.6514** | 0.0495*** | 0.9857*** | 0.0448*** | 0.7524** |
| | 0.5 | 0.0448*** | 1.1714*** | 0.0795*** | 2.833*** | 0.0881*** | 3.6048*** |
| | 1 | -0.152 | 0.1429 | 0.01 | 0.5762** | 0.0086 | 0.3857 |
| K | 0.01 | 0.0333*** | 1.2857*** | 0.0495*** | 2.7286*** | 0.0404*** | 1.7333*** |
| | 0.1 | 0.0014 | 0.0714 | 0.0005 | 0.7476** | 0.0076 | -0.1095 |
| | 1 | -0.0395 | -1.2857 | -0.0143 | -0.1952 | -0.0616 | -1.5181 |

* - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$

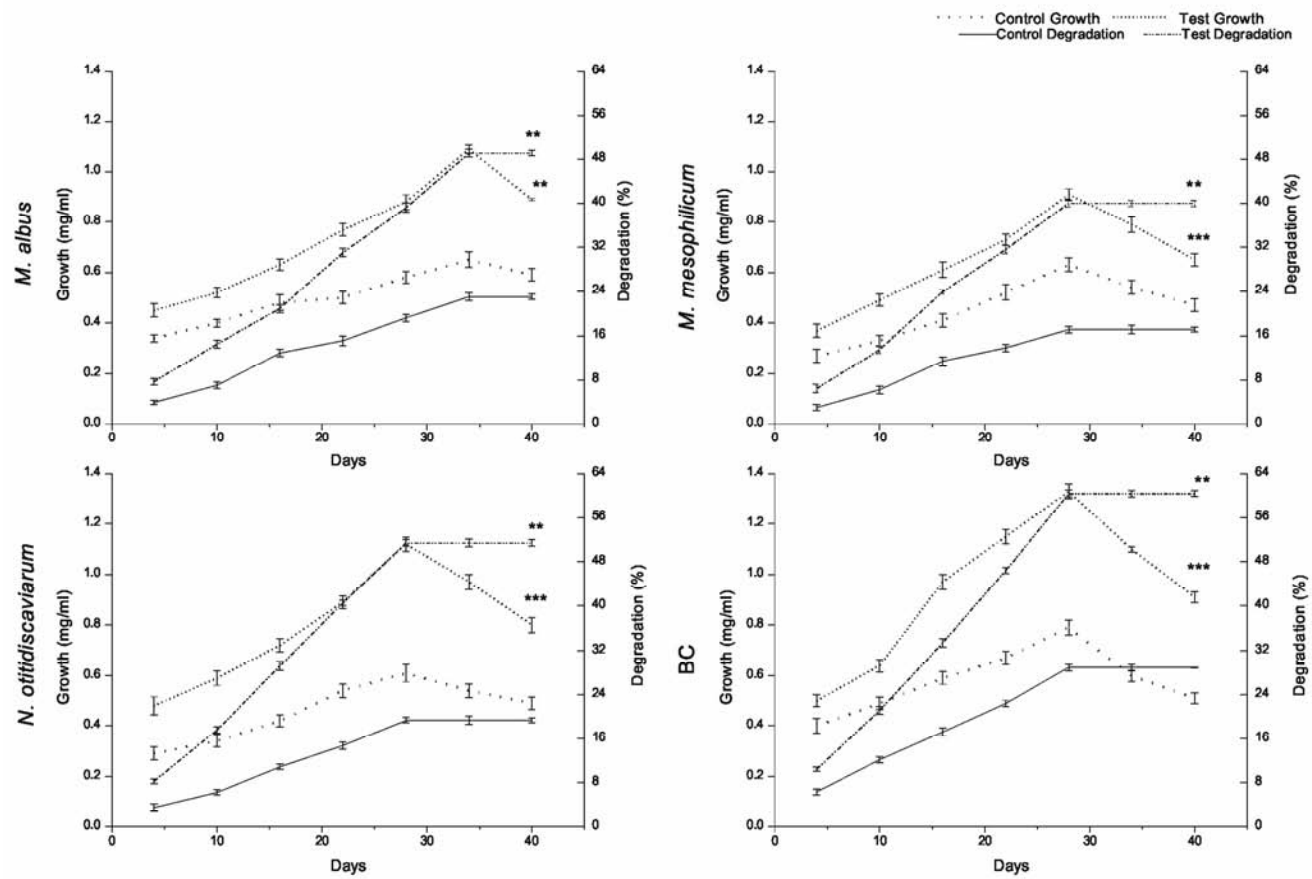


Fig 4—Effect of optimized conditions on growth and degradation by *M. albus, M. mesophilicum, N. otitidiscaviarum* and a BC (*** - $P \leq 0.001$, ** - $P \leq 0.01$)

degradation with optimized conditions was with a BC, maximum effect in increase in growth was by a BC (0.54 mg/ml) whereas, maximum effect in degradation was by *N. otitidiscaviarum* (32.1%) The data of bioremediation studies as analyzed by Paired T-test revealed that growth was highly significant at $P \leq 0.001$ except in *M. albus* ($P \leq 0.01$) whereas, degradation was significant at $P \leq 0.01$ (Fig 4).

## Discussion

Nutrients are one of the major factors limiting hydrocarbon biodegradation in soil as well as in sea. Addition of nutrients as fertilizers resulting in increased oil biodegradation has been reported by several workers[10-15]. Present study supports the above observation of enhanced effects in growth and degradation with addition of N, P and K fertilizers. The observation of addition of excess K (above 0.1%) which proved to be toxic is in accordance to the results of Kenelly *et al*, 2002 who reported that overloading oil contaminated sea water or sediments with nutrients inhibits microbial activity due to its toxic effect[16]. Kenelly et al, 2002[16] have reported that as crude oil is a complex mixture of hydrocarbons, a consortium can more effectively degrade it rather than a single isolate as the organisms complement each other through interdependence. The interaction among a consortium may also be necessary for the initial step in the conversion and later for the transformation or mineralization of the compounds. Results of a bacterial consortium exhibiting maximum growth (1.33 mg/ml) and degradation (60.4%) supports the above observation.

After 40 days of incubation, *M. albus* showed 50.9% of residual hydrocarbon, *M. mesophilicum* of 60%, *N. otitidiscaviarum* of 48.6% and a BC of 39.6%. Complete mineralization could not be achieved within this period. Groß *et al*, 1999 have reported that 10-30% of the residual hydrocarbon remains when bioremediation treatment is applied, but when the treatment time was prolonged, increase in degradation has been observed[17]. In the present study

the treatment could not be prolonged beyond 40 days to substantiate this observation. The reasons for low ultimate degradation rates include low bioavailability of contaminant, the accumulation of recalcitrant compounds, inhibiting metabolites and the lack of microbial growth factors[18]. Weathering of oil includes evaporation, dissolution, water-in oil and oil-in-water emulsion, photo-oxidation etc. that depend on environmental conditions and availability of sunlight. As the experiments were carried out at microcosm level on an environmental shaker in the laboratory, weathering was found to be insignificant.

Inorganic nutrients as N, P and K severely limit the extent of hydrocarbon degradation in marine environment. Present study concludes that addition of optimum concentrations of N, P and K enhanced biodegradation. Population interactions are of paramount importance in natural consortia, especially when considering biodegradation of complex substrates as crude oil. A defined consortium of three strains of *M. albus*, *M. mesophilicum* and *N. otitidiscaviarum* to had been used to simplify interactions in the consortium due to its broader enzymatic capability that proved to be more effective. Hence, nutrient addition can be used to evolve a successful cost-effective *in situ* bioremediation strategy for restoration of oil contaminated marine environment.

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