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KINETICS AND BIODEGRADATION OF HEXACHLOROCYCLO-HEXANE ISOMERS BY MIXED BACTERIAL CULTURES

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ABSTRACT

Effect of temperature on the growth of consortia AHR and GHR as alpha-HCH was studied. Both the consortia showed growth at all temperatures, the maximum growth being at 30° C. Acidic pH reduced the alpha-HCH degradation more drastically than by alkaline pH with the addition of auxilliary co-substrates such as glucose. It was found that the degradation rate of alpha-HCH was faster than that without glucose. Higher maximum specific growth rate (Hmax) and lower saturation constant values indicated that the lower concentration of alpha–HCH can be degraded completely and at higher concentrations the reduction in the rate of degradation may be due to substrate inhibition.

INTRODUCTION

Increased and indiscriminate use of pesticides in modern agriculture and public health has caused air, water and soil pollution. Hexachlorocyclohexane (HCH) is one of the organochlorine insecticides extensively used in India which amounts to more than 40% of total pesticides used. Theoretically HCH contains mainly alpha-, beta-, gamma and delta-isomers. The pollution problem with HCH is mainly due to the non-insecticidal alpha-, beta- and delta-isomers rather than lindane (gamma-isomers). Developed countries have therefore restricted the use of lindane and banned the technical mixture. Still they have HCH residue problem persisting although theoretically, gamma-isomer should get degraded. There are only a few research workers, who have reported the inter conversion of lindane to other isomers in soils, plants, animals, insects, water etc. at high temperature by UV-radiation (Deo et al. 1994).

Pesticides are generally applied to the soil, plants, water bodies and human settlements either as liquids, dusts or granules. It has been computed that only less than 1% of the total pesticides applied reaches the target organisms while more than 99% enter the ecosystems (Pimental and Levintan, 1986). Due to their lipophilic nature they get accumulated in bio-concentrated form in the lipid and fat tissues of the living systems at different stages of the food chain and eventually reach human body.

Pesticide consumption in India has shown a significant from 32 g ha^{-1} in 1954 to 336 g ha^{-1} in 1980 (Mrinalini, 1986) and presumably, it might have gone up much higher now. After DDT was legally restricted for use in many countries, hexachlorocyclohexane became more important as a substitue for DDT. Lindane, the active principle in HCH (known as gamma-HCH), disappears relatively more rapidly than DDT.

The major sources of empidemics of accidental poisoning of pesticides have been the contamination of food by pesticide formulations during transport or storage and the use of pesticide treated grains as food. The epidemics have been reported in several

countries of the world. In Japan deaths of several people have been reported after eating bread made from contaiminated flour. Major mass poisoning incidences in India are presented in Table 1. The adverse effect of pesticides on non-target organism have been reviewed by number of researchers. (Agarwal 1986 and Sethunathan 1977). Pesticides are suspected to be the cause of hazards such as birth defects, cancer, still births, neurological disorders that damage to brain, and sterility (Hurtado 1980). Mwanza (1987) reported that in Zambia that 90% of the population is exposed to DDT poisoning through fish, which is a predominant dietary component.

Whaetstone et al. (1953) reported that by heating HCH isomers in a sealed tube with anhydrous $FeCl_3$ and Frilled Craft Ctalysts Symetrical beta-isomer was readily converted to the alpha-isomers. The gamma-isomer mostly got isomerized to the delta-isomer with small quantities of alpha-isomers, while the delta isomer was found to give mostly alpha-and small quantities of gamma-isomers. Kamada (1971) and Ishikuva (1972) reported the interconversion of HCH isomers in soil, crops and tissues of living organisms. Newland et al. (1969) found both alpha-HCH and delta-HCH in aquatic sediments incubated with pure gamma-HCH.

Benezet and Matsumura (1973) have reported that microorganisms are capable of procuring alpha-HCH from gamma-HCH both in laboratory and in aquatic sediments. They isolated Pseudomonas putida strain from soil which converts gamma-HCH to alpha-HCH. Further, their results reveal that presence of high levels of alpha-HCH and beta-HCH in the environement may result in isomerization of gamma-HCH leaving behind residues of other constitutents of HCH. Barik (1984) and Khan (1980) have reviewed the various methods that have been tried to eliminate the levels of pesticide residues from soil. Each of these methods have their own limitations from the point of view of field applications.

Mac Rae (1989) and Haider (1979) have conducted studies on biotransformations of HCH-isomers by aerobic and anaerobic soil micro-organisms. Their results reveal that gamma-HCH was the most easily degraded isomer while alpha-, beta- and delta-isomers were slowly dechlorinated. Heritage and Mac Rae (1979) quoted that the most active bacyerium identified is *Clostridium sphenodies* which metabolizes alpha-and gamma-HCH under anaerobic conditions. The results reported by Bachmann et al. (1988) reveal that alpha-HCH was degraed more easily under acrobic than methanogenic conditions in heavily contaminated soil slurries and the beta-HCH was recalcitrant to biodegrdation. They also showed that the factors influencing the aerobic degradation of alpha-HCH in soil slurries were temperature, auxillary carbon source, substrate concentration and homogeneties of soil. Temperature in the range of 20° to 30° C was found to be more favourable for the degradation. Heritage and Mac Rae, (1979) showed that the optimum pH and temperature for the degradation of gamma-HCH by C. Sphenoides were 8.0 and 40° C, respectively. Further, the isoloation of Pseudomonas from sugarcane rhizosphere soil that readily degraded alpha-, beta- and gamma-isomers of HCH under anaerobic conditions (Sahu et al., 1990).

Since the natural process of degradation of these pesticides take a longer period, it has become necessary to accelerate the process by suitable methods. Biodegradation is considered to be a potential means of elimination of some organic pollutants. It has been

Table 1. Effect of pH on Degradation of Alpha HCH (25 mg L^{-1})

Time in Days		1		2		4		6		8		10		12		14	
Initial pH	Final pH (After 14 days)	Growth mg L^{-1}	Cl^{-1}	Growth mg L^{-1}	Cl^-	Growth mg L^{-1}	Cl^-	Growth mg L^{-1}	Cl^-	Growth mg L^{-1}	Cl^-	Growth mg L^{-1}	Cl	Growth mg L^{-1}	Cl	Growth mg L^{-1}	Cl
4.0	4.19	13	ND	18	ND	21	ND	26	ND	24	ND	22	ND	21	ND	18	ND
5.0	5.12	25	ND	31	ND	36	ND	42	ND	39	ND	37	ND	34	ND	31	ND
6.0	5.71	29	ND	33	ND	45	ND	60	ND	61.5	ND	57	8.95	53	17.81	45	30.88
7.0	6.72	26	ND	30	ND	37	10.24	49	22.81	51	34.16	46	42.89	41	55.55	36	62.81
7.5	7.33	27	ND	32	15.3	38	22.46	41	43.92	48	51.76	45	64.10	42	72.38	39	80.95
8.0	7.58	38	ND	43	3.5	52	15.62	57	23.32	59	29.68	62	35.41	54	42.72	47	53.06
9.0	7.91	40	ND	48	ND	54	13.67	59	25.44	63	30.72	65	34.41	67	39.88	63	46.62
10.0	8.02	42	ND	51	ND	57	16.13	64	21.68	67	28.68	70	33.92	73	41.46	68	47.84

ND – Not Detcted.

observed that native micro-ogranisms such as Pseudomonas spp. are capable of degrading only one or very few structurally similar chemicals (HCH isomers). This leads to the isolation HCH degrading microbial strains which can effectively degrade higher levels of HCH isomers under varying environmental conditions.

In view of the above, in the present study an attempt has been made to endue a suitable method to eliminate Hexachlorocyclohexane isomers using microbial consortia developed in the laboratory for the degradation of different HCH isomers. Further, influence of various physico-chemical parameters on the degradation of HCH isomer was also studied.

EXPERIMENTAL PROGRAMME

Chemicals

In the present study HCH obtained from Hindustan Insecticides Ltd., Mumbai, alpha- and gamma- isomer of HCH from Aldrich Chemical Company, Milw, U.S.A. and beta and delta-isomers from Central Food Technological Researarch Institute (CFTRI), Mysore were used. All other chemicals, reagents, solvents and media chemicals were obtained from Ranbaxy Chemicals Ltd., Mumbai, India.

Microbial consortia and mineral composition

Microbial consortia like A-HCH-C, G-HCH-S, AHR, BHR, GHR and DHR isomers were obtained from CFTRI, Mysore have been used. The basal mineral medium used for growing microbial consortia consists of KH_2PO_4–2.72 g; $(NH_4)_2\ SO_4$–0.50g; $Na_2\ HPO_4$–3.5 g; $MgSO_4.\ 7H_2O$–0.20 g; Ca $(NO_3)_2$–0.10 g and trace element solution - 1.00 ml. The pH of the medium was maintained in the range 7-7.5.

Reagents for protein estimation

Reagent A : 2% of Na_2CO_3 in 0.1 N NaOH

Reagent B : 0.5% $CuSO_45H_2O$ in 1% sodium and potassium nitrate

Reagent C : By mixing 59 ml of reagent A + 1ml of reagent B

Reagent D : 1 part of Folin - Ciocalteau reagent + 2 parts of distilled water.

Development and Maintenance of Microbial Consortia

In this study microbial Consortia viz. A-HCH and G-HCH-S were developed in the laboratory by a long term enrichment of a mixed sample of pesticide contaminated soil and sewage and HCH contamination as a fermented vegetable, respectively (Chandrashekaraiah, 1993). The consortium AHR was obtained by further enriching A-HCH-C in shake flasks containing alpha-HCH as the sole carbon source. The consortium readily utilized alpha-HCH as the source of energy. Consortia BHR, GHR and DHR were developed by similarly enriching a mixture of A-HCH and G-HCH-S using beta, gamma and delta isomers, respectively as sole carbon source. The substrates, alpha-, beta-, gamma- and delta-isomers of HCH were added to the medium as a sole carbon source as solutions in acetone for the flasks to be getting dried, later acetone was evaporated completely before the addition of mineral solution.

Analysis of Consortia for Microbial Growth

Consortia AHR, BHR, GHR and DHR were grown as sterile medium containing 25 mg L^{-1} of alpha-HCH, beta-HCH, gamma- and delta-HCH, respectively. Samples from

flasks were grown for 14 days in a rotary shaker at 30°C with pH maintained at 7.5. They were later serially diluted and plated on a Luria broth-agar for the analysis of bacterial strain. Different types of colonies were characterized on the basis of colony morphology.

Further, preinoculum of microbial consortia (1ml) from stored culture was transferred to 50 ml sterile medium containing respective substrate in 250 ml conical flasks and incubated on a rotary shaker running with a speed of 150 rpm, at 30° C. Culture was allowed to grow till it reached mid exponential phase (3 to 4 days). Cells were then harvested by centrifugation for 10 minutes at 8000 rpm. One ml of this suspension was used as inoculum.

For optimization of pH, different pH values of the medium were adjusted by varying the proportion of KH_2PO_4 and Na_2HPO_4. pH values below 5.5 and above 8.0 were adjusted with addition of 1N HCl and 1N NaOH solutions, respectively. Also, different co-substrates were added to the medium as a auxillary source of carbon along with HCH. Co-substrates like glucose, cellulose and sawdust were added at 100 mgL^{-1}, 1000 mg L^{-1} and 1000 mg L^{-1}, respectively as four incremental doses of one fourth of the amount on 0, 2, 4 and 6 days after sample inoculation, and samples were withdrawn at intervals for further analysis.

Growth Estimation

The growth of consortia was determined by measuring turbidity at 550 nm using spectrophotometer (Schimadzu UV-160 Å, Japan) or by estimating the total protein by the following method. A known quantity of culture cells were harvested by centrifugation at 8000 rpm for 10 minutes and resuspended in 3.4 ml of distilled water to which 0.6 ml of 20% NaOH solution was added and shaken. Test tubes were incubated in a boiling water bath for 10 minutes and after cooling 0.5 ml of these hydrolysates were taken for protein estimation as per the method of Lowry et al. (1951).

Determination of Residual Substrates

Extraction :

50 ml of culture has been taken in a separating funnel to which 150 ml of hexane was added and shaken throughly for five minutes and the hexane layer was collected. The extraction has been carried out thrice and the filtrates were pooled. This has been passed through Na_2SO_4 to remove moisture and evaporated to required levels (Hortwitz 1980).

Detection and quantification of alpha-HCH :

Thin layer chromotography (TLC) technique has been used for the detection and quantification of alpha-HCH residues (Sharma 1973).

Estimation of inorganic chloride :

For 2 ml of known sample, 0.2 ml of 0.25 N ferric ammonium sulphate and 0.2 ml of mercuric thiocyanate solutions were added. The colour developed was measured at 460 nm using UV-160 spectrophotometer as used in growth estimation. Calibration curve was prepared using NaCl as standard to compute the results (Bergmann and Sanik 1957).

RESULTS AND DISCUSSION

Effect of Temperature on Growth of Consortia AHR and GHR

Effect of temperature on the growth of consortium AHR on alpha-HCH and consortium GHR on gamma-HCH, under stationary condition was studied (Figs 1 &2). The result

obtained reveals that both the consortia contain mesophilic micro-organisms which showed favourable growth at ambient temperature (30°C). It is also observed that both the consortia have exponential growth upto 48 hr beyond which declining of growth rate was observed. At 54° C both the cultures have showed an initiation of growth but failed to pickup after two days. Bechmann et al. (1988) have demonstrated that mesophillic range of temperature to be optimal for degradation of alpha-HCH in the soil slurry, the degradation being maximum at 30° C. Further, anaerobic degradation of gamma-HCH in moist soil was found to be fastest at 35°C (Bhuyan et al. 1993).

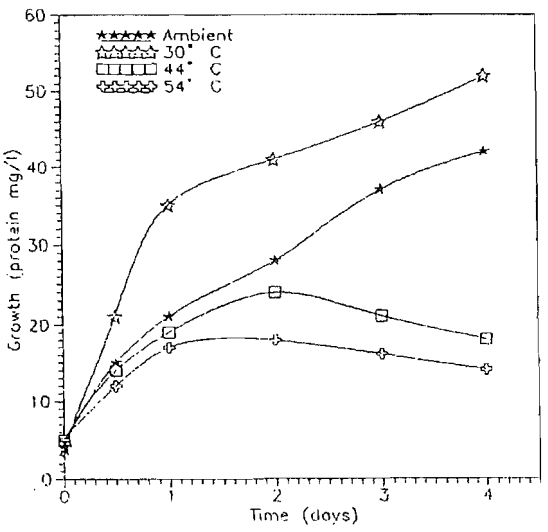

Fig. 1. Effect of temperature on growth of consortium AHR

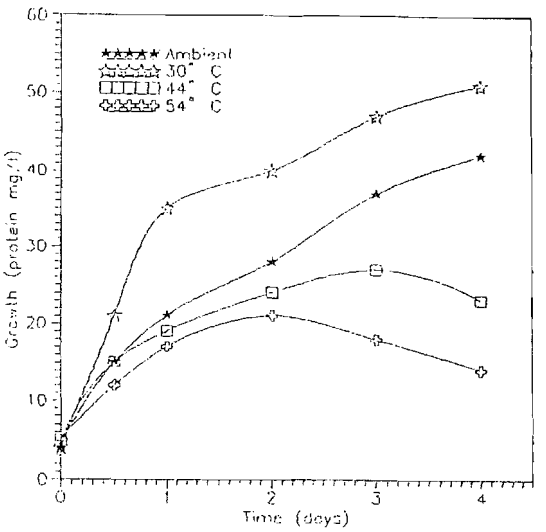

Fig. 2. Effect of temperature on growth of consortium GHR

Effect of pH on Degradation of Alpha-HCH

Effect of pH on the degradation of consortium AHR as alpha-HCH was studied and the results are presented in Table 1. The above Table reveals that during 6th day the growth was minimum at pH 4.0 (26 mg). At higher pH (8.0 and 10.0) the total biomass formed was higher than at any other pH. However, there was no correlation between the total biomass formed and degradation of alpha-HCH as evidenced by Cl^- release. A pH values between 6.0 and 7.5 Cl^- release was shown to be increased while at pH values between 7.5 and 10 release of Cl^- was only marginal. After 14 days maximum release of Cl^-, about 80%, was observed with an optimum pH of 7.5. Further, it was also observed that at pH 6.0 hardly 31% Cl^- was observed after 14 days of growth, whereas at pH 7.0 and 8.0 the Cl^- release was 63% and 53% respectively. This inferred that pH play critical role for alpha-HCH degradation especially when the shift is towards acidic side.

Effect of Glucose as an Auxilliary Carbon Source

Effects of addition of glucose as single and split does were studied on 25 mg L^{-1} alpha-HCH by consortium AHR. Growth and degradation of 25 mg L^{-1} alpha-HCH with time when glucose was incrementally fed or initially fed at a time are plotted in Fig. 3. Higher biomass formation was observed in the case of glucose added cultures as compared to the control containing only alpha-HCH. Single addition of glucose accelerated growth upto first four days but later retain almost stationery, whereas in the case of addition of split does a steady increase in growth upto the twelth day was observed (Figure 3 A).

A steady decrease in the alpha-HCH level in the medium was observed (Fig. 3 B). Complete degradation of alpha-HCH was observed on 8th day in the case of glucose added cultures, whereas in the control alpha-HCH disappeared during 10th day. The mode of addition of glucose (single dose or four split doses) showed significant effect on the degradation of alpha-HCH. A significant difference in Cl^- release was observed between control (without glucose) and the cultures where glucose was added as auxiliary substrate. However, the mode of addition of glucose did not show any significant impact on the chloride release. Both the cultures showed a release of about 90% Cl^- after 14 days.

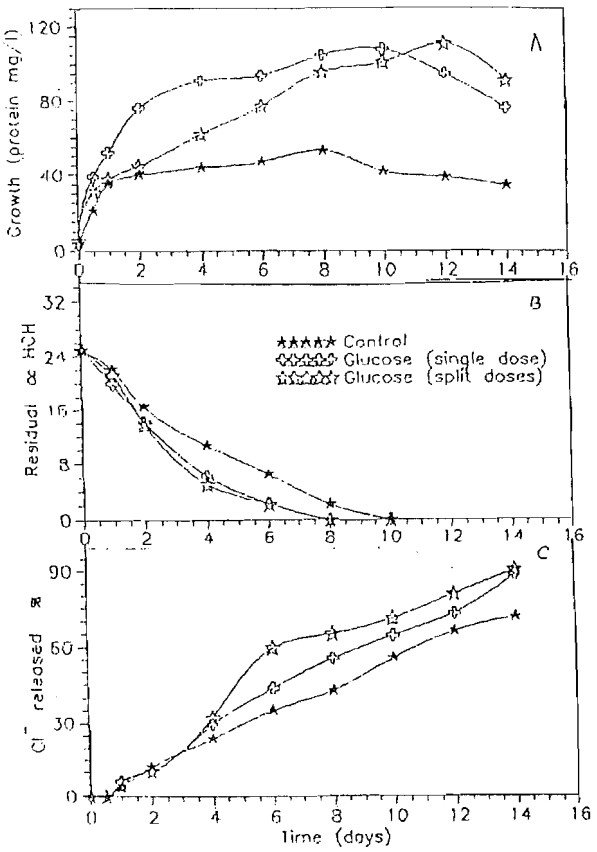

Fig. 3. Growth and degradation of 25 ppm alpha-HCH when glucose was incrementally fed or initially at a time as a co-substrate.

Determination of Biokinetic Parameters

The effect of substrate concentration and the rate of growth in declining growth phase is well described by Monod's equation.

$$\mu = \mu_{max} \frac{S}{S+K_t} \quad \text{.....(1)}$$

In many instances it has been found difficult to determine the biokinetic parameters μ_m and K_s accurately using Monod's equation. Therefore, Lineweaver and Burk rearranged the equation (1) to faciliate linear plot which enables more exact determinations of μ_m and K_s

$$\frac{1}{\mu} = \frac{1}{X}\frac{dX}{dt} \quad \text{.....(2)}$$

$1/\mu$ versus 1/S for different substrate concentrations were plotted and a typical plot so obtained is shown in (Fig. 4 B). The maximum specific growth rate and Monod's saturation constant values (K_s) for different alpha-HCH concentrations are presented in Table 2. From this Table it is evident that μ_{max} values show a decreasing trend when the K_s values were gradually increased. The decreasing values suggest the possibility of repression of enzymes necessary for the growth. Further, it is also reflected that the increase in K_s values signify a decreasing affinity for the substrate when the initial substrate concentration is increased.

Table 2. Comparision of Specific Growth Rate Values

Sl. No.	Alpha-HCH Concentration mg L^{-1}	Maximum Specific Growth Rate Per Day	
		Monod's Equation	Logistic Equation
1.	5	0.469	0.356
2.	10	0.390	0.398
3.	25	0.324	0.340
4.	50	0.214	0.225

Logistic equation

The rate of increase in biomass concentration is a function of the biomass only, i.e.

$$\frac{dX}{dt} = f(X) \quad \text{.....(3)}$$

Such a form does not require to reflect changes occuring in the medium during growth. One simple generalised model equation derived by Mathus is

$$f(X) = \mu X \quad \text{.....(4)}$$

The logistic equation has been known for many years. The classical S-shaped curves generated when a population growth may be empirically described as

$$\frac{dX}{dt} = \mu_m X \left(\frac{1-X}{X_{max}}\right) \quad \text{.....(5)}$$

$$\frac{dX}{dt} = \mu_m \frac{X(X_{max}-X)}{X_{max}} \quad \text{.....(6)}$$

Eq. 4 describes the change in population density during exponential growth phase and as well the equilibrium stage growth phase. Eq. 5 differs from Eq. 4 by the term

($1\text{-}X/X_{max}$) which provides restriction factor absent from the basic growth Eq. 4 and enables a reduction in the rate of change of biomass concentration to a zero value to be modelled. At the begining of the batch growth process, when the biomass concentration is small, all nutrients are in excess and conditions are essentially those required for unlimited exponential growth, then X/X_{max} is small and the term ($1\text{-}X/X_{max}$) tends towards unity and Eq. 5 reduces to Eq. 4. As X increases, X/X_{max} tend towards unity and ($1\text{-}X/X_{max}$) becomes significantly lesser than one. Since μ_m is the reducing factor which effectively decrease the μ_m value of thereby restricting the rate of increase in the microbial growth phase, $X = X_{max}$, hence ($1\text{-}X/X_{max}$) = 0 and $dX/dt = 0$ which is charactristic of the equilibrium growth phase.

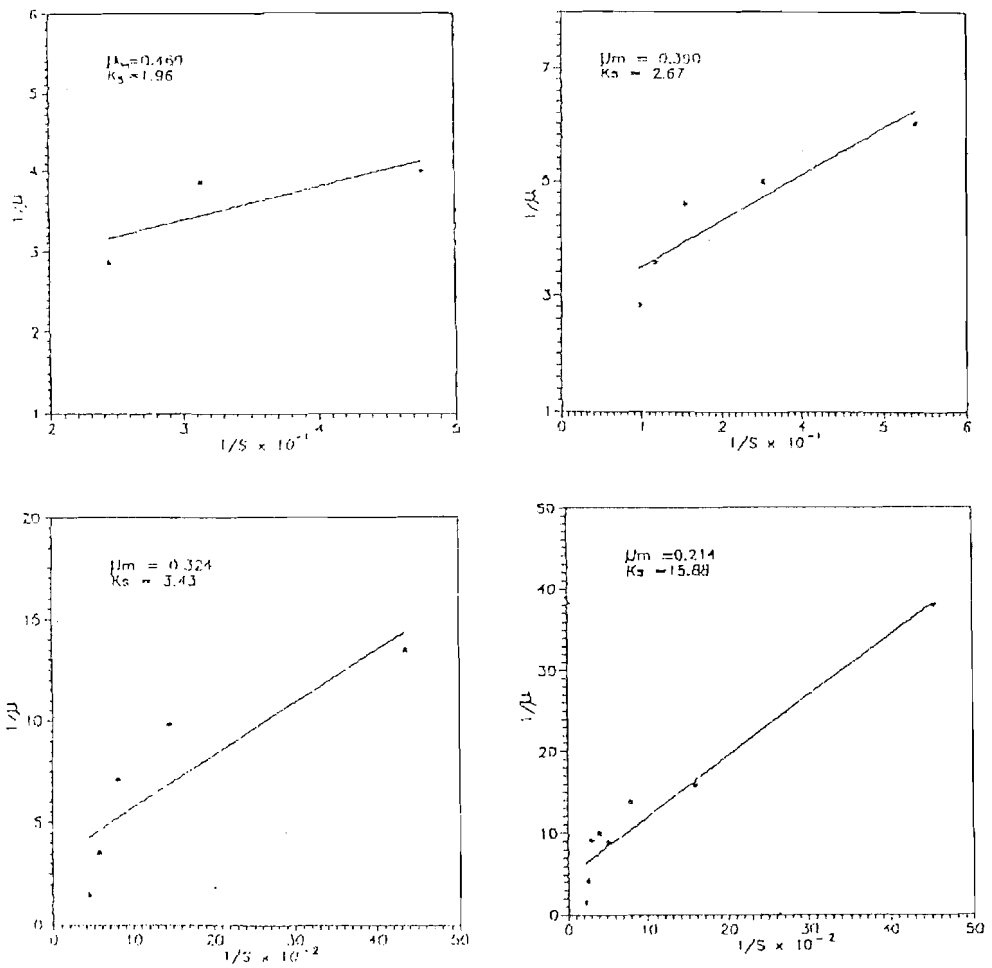

Fig. 4. Plot of reciprocal of specific growth rate versus reciprocal of substrate concentration

This simple model frequently describes microbial growth. However, it must be recognised that there are likely to be many factors other than X_{max} which contribute to the limitation of microbial growth size. Further, Eq. 6 has been rearranged to facilitate linear plot which enables determination of μ_m.

$$\frac{1}{X}\frac{dx}{dt} = \mu_m\left(\frac{X_{max}-X}{X_{max}}\right) \qquad \text{.....(7)}$$

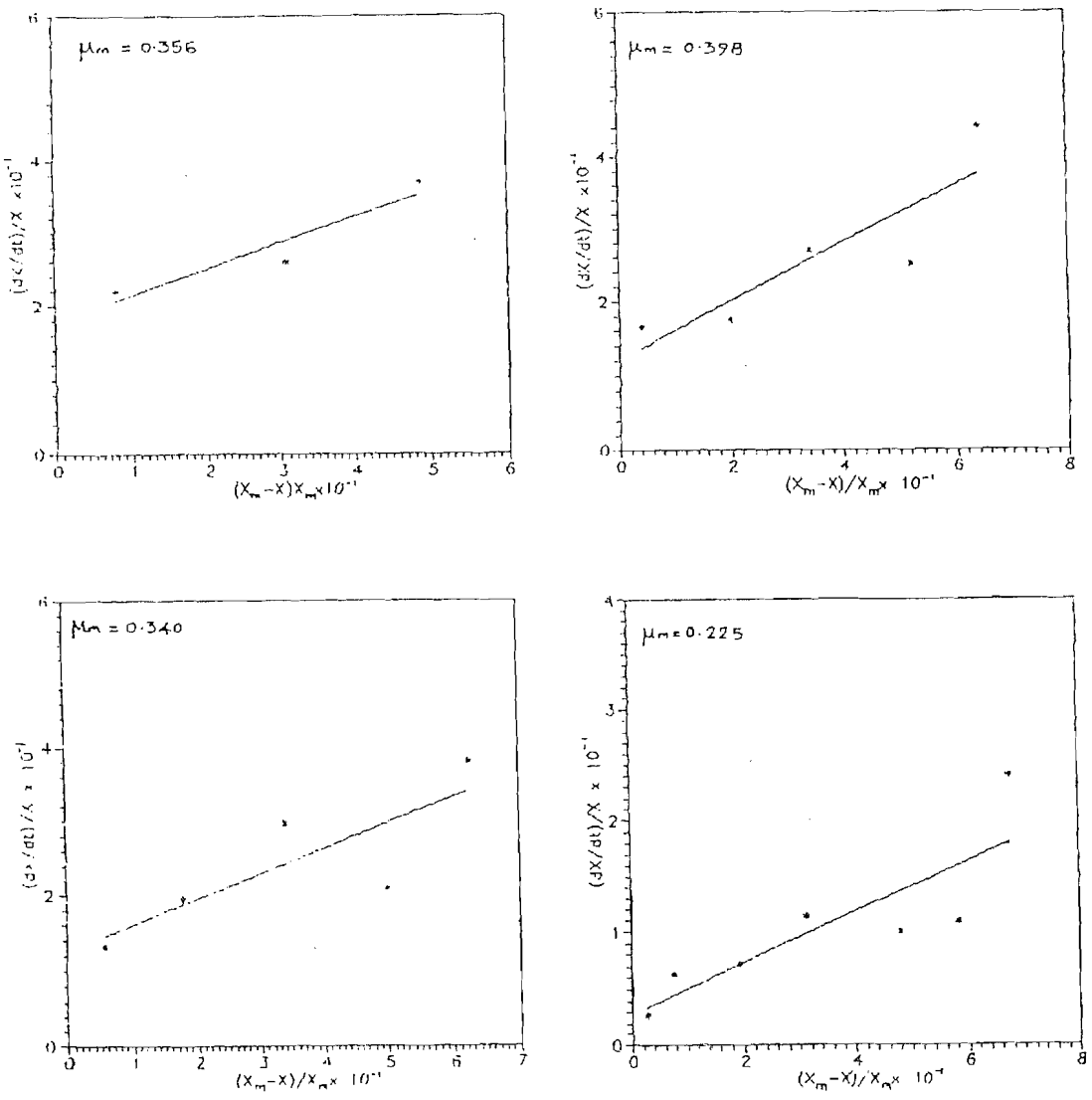

Fig. 5. Plot of 1/X (dX/dt) versus $(X_{max}-X)$ / X_{max}

Typical plots of (1/X) (dX/dt) versus $(X_{max} - X)/X_{max}$ are shown in Fig. 5. The slope of straight line gives values for different alpha-HCH concentrations. Maximum specific growth rate values obtained by using Monod's and Logistic equations are also presented in Table 2. The differences in maximum specific growth rate values by both the methods are due to differences in basic dependency of over specific experimental variables. Monod's equation relates to substrate concentration, whereas logistic equation relates to biomass concentration. However, the differences are negligible and the validity of either model for the experimental data is evident.

CONCLUSION

The consortia AHR and GHR showed ability to grow at wide range of temperatures between 25°C and 55°C, though both had a optimal temperature of 30°C. Further, the degradation of alpha-HCH by consortium AHR was optimal at pH 7.5. Acidic pH reduced the degradation more drastically than by alkaline pH. Addition of auxiliary carbon source such as glucose showed improved degradation of alpha-HCH.

Maximum specific growth rate from Monod's and Logistic equations have shown similar trend and the values are in the range of 0.125 to 0.469 per day and K_s values are in the range of 1.96 to 15.85 mg L^{-1}. Higher and lower K_s values indicate that the lower concentration of HCH can be degraded completely, and at higher concentration reduction in the rate of degradation may be due to substrate inhibition.

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