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Optimization of carbon sources for the preparation of inoculum of hexachlorocyclohexane-degrading microbial consortium

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Many simple and complex carbon sources were screened along with technical grade hexachlorocyclohexane (tech-HCH, 25 ppm) for their synergistic effect on the growth of HCH degrading microbial consortium. Highest biomass production was in molasses followed by glucose, sucrose, rice straw extract supplemented with glucose, rice straw hydrolysate, nutrient broth and wheat bran hydrolysate (WBH). However, the inoculum grown on WBH and 25 ppm HCH showed best ability to degrade HCH. Nearly 80 to 90% of all the 4 isomers of HCH disappeared within 72 h of incubation. This is followed by molasses-HCH grown inoculum, which showed 65 to 70% degradation. Microbial consortium grown for 72 h in WBH containing 0.75% reducing sugar and 25 ppm HCH was efficient to degrade HCH residues in liquid medium.

Keywords: Adaptation, Carbon sources, Consortium, HCH-degradation, Microbes

Hexachlorocyclohexane (HCH) is one of the most extensively used organochlorine pesticides. The commercial formulation of HCH contains a mixture of α-, β-, γ-, δ- and other isomers of which only γ-isomer has insecticidal properties. Other isomers are highly recalcitrant and persist in the soil (Spain and Veld 1983).

HCH-degrading consortium, developed in our laboratory was found to degrade all the major isomers of HCH under laboratory conditions. However, expression efficiency of this attribute largely depends upon the size and colonization of the ecosystem by the consortium (Manonmani et al 2000). Application of the inoculum to larger areas would need mass production of the consortium. Since only limited quantities of HCH can be used as substrate, the biomass build up with HCH as sole source of carbon and energy is rather low. Hence, it is necessary to use other easily utilizable carbon sources as co-substrates for the preparation of bulk inoculum or without losing the degrading ability. The objective of this work was to characterize the effective carbon source and optimize the preparation of inoculum without loss in biodegradative ability.

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Materials and methods

Chemicals, sources of carbon and medium: Technical grade HCH containing α-(60-70%), β-(5-12%), γ-(10-15%) and δ-(6-10%) was obtained from Hindustan Pesticides Ltd. Mumbai, India. The minimal medium used in degradation studies contained (mg/ml), KH_2PO_4,0.675; Na_2HPO_4, 5.455; NH_4NO_3, 0.25; $MgSO_4$. $7H_2O$, 0.2; $Ca.(NO_3)_2$, 0.1 and 1 ml mineral solution containing, (mg/ml) $FeSO_4.7H_2O$, 1; $MnSO_4.H_2O$, 1; Na_2MoO_4. 0.25; H_3BO_3, 0.1 and $CuCl_2.2H_2O$, 0.25. Added H_2SO_4, 5ml. The pH of the medium was 7.5 (Manonmani et al 2000).

Simple and complex carbon sources used as co-substrates for biomass build up are listed in Table 1. Bagasse (juice extracted sugarcane stem), corn-cob and rice straw were procured from local market. Wheat bran was obtained from the Department of Flour Milling, Baking and Confectionery Technology, CFTRI, Mysore. Molasses and corn steep liquor were purchased from Excise Department, Government of Karnataka. All other chemicals including acetate, succinate, sucrose, and glucose were of AR grade.

Microbial consortium: The microbial consortium capable of degrading all the four isomers of HCH was by long-term enrichment of HCH-contaminated soil and sewage in a semi-continuous column reactor followed by enrichment in shake flasks using different isomers of HCH separately as sole source of carbon and energy. The consortia thus obtained had the capacity to degrade 100 ppm of α-HCH, 400 ppm of γ-HCH, and 25 ppm each of β- and δ-HCH, respectively in shake flasks (Manonmani et al 2000). These four consortia were pooled together and inoculated to 10 ppm HCH and acclimatized to higher concentrations of HCH.

Degradation of HCH: Degradation

experiment was carried out in 250 ml Erlenmeyer flasks. Required quantity of HCH dissolved in 50 µl acetone was placed at the bottom of sterile, dry Erlenmeyer flasks under aseptic condition and allowed to evaporate the acetone. Added 50 ml minimal medium, inoculated the required consortium and incubated in a rotary shaker at 26-28°C. Samples were drawn at regular intervals and analyzed for growth in terms of total protein (3), chloride released and residual substrate. Both abiotic and biotic controls were also maintained during all the experiments and each test/experiment was triplicated.

To study the effect of co-substrates on biomass build-up and degradation of HCH, co-substrates were added at 1.5% sugar level (as glucose) to minimal medium along with 25 ppm of tech-HCH. HCH degrading consortium was inoculated to these flasks and incubated for 72 h on a shaker at 26-28°C. The cells were harvested by centrifugation at 15,000 g for 10 min, washed with sterile minimal salts medium and inoculated to flasks for degradation of HCH. The optimum sugar concentration required for maximum biomass production without loss in HCH-degrading ability was obtained by growing the HCH-degrading consortium in varying concentrations of sugar along with tech-HCH for 72 h. The cells were harvested, washed and tested for degradation of HCH.

Extract of rice straw was prepared by cooking finely powdered rice straw in de-ionized water at 1:50 ratio for 15 min with constant stirring. The extract obtained was neutralized with NaOH and centrifuged. For hydrolysates of wheat bran, corn-cob, bagasse and rice straw 6N H_2SO_4 was used instead of water.

Protein estimation: Growth was estimated as protein of the alkali digested culture broth. To 1 ml culture broth, 2.4 ml distilled water and 0.6 ml of 20% NaOH were added to make a final volume of 4 ml and digested in a boiling water bath for 10 min. From this digest, 0.5 ml was used for protein estimation by Lowry's method (1951).

Chloride estimation: Chloride was estimated by the modified HNO_3-$AgNO_3$ method (Bidlan and Manonmani 2002). One ml of culture broth was centrifuged and the supernatant was taken in a test tube. The cells were washed with 0.1N NaOH (50 µl) and 950 µl of minimal medium. All the supernatants/washings were pooled, 1 ml of 0.15N HNO_3 and 1 ml of 0.1N $AgNO_3$ were added with mixing at each step, allowed to stand for 20 min at room temperature and measured the O.D at 600 nm. The amount of chloride was computed from a standard curve prepared for NaCl. Reducing sugar was estimated by dinitrosalicylic acid method (Miller 1959).

Residual substrate estimation: Residual substrate from the acidified culture broth (pH 2.0) was extracted thrice with equal volumes of ethyl acetate. The ethyl acetate fractions were pooled, concentrated and passed through florisil columns. The fraction containing the residual substrate was concentrated and used for thin layer chromatography (TLC) and gas chromatography (GC).

TLC was done by loading samples on silica gel plates, which were developed in cyclohexane. The residual HCH spots were identified and measured after spraying the air-dried developed plates with 2% o-tolidine in acetone. The residual substrate spots were delineated by marking with a needle and the area was measured. The concentration was computed from a standard plot of log concentrations *versus* square root of the area, prepared for the standard HCH.

The ethyl acetate layer, containing residual HCH was evaporated to dryness and after appropriate dilution with acetone, it was injected into gas-chromatograph (Fisons 8000) equipped with a ^{63}Ni electron capture detector and stainless steel-column (200 mm x 2 m) packed with 1.5% OV-17 plus 1.95Q F-1 on Chromosorb W 80-100 mesh. The column, injector and detector were maintained at 230, 230 and 320°C, respectively with a flow rate of carrier gas (nitrogen) at 50 ml/min. Under these conditions, the retention time was found to be 3.34 min for α-HCH, 4.43 min for β-HCH, 3.98 min for γ-HCH, and 5.1 min for δ-HCH. The recovery of the four HCH isomers ranged from 92 to 95% from the mineral salts medium.

Enumeration for viable counts: The survival or proliferation of individual members of the consortium for viable cell population was determined by plating (Sahu et al 1996).

Results and discussion

The HCH-degrading consortium was able to degrade 100 ppm of α-isomer, 400 ppm of γ-isomer and 25 ppm each of β- and δ-HCH isomers (Manonmani et al 2000). When all these four consortia were pooled and inoculated to tech-HCH, which was a mixture of all the four isomers, the consortium got established and was found capable of degrading HCH. The characterization of the consortium revealed that it comprised of 10 bacterial species containing 7 *Pseudomonas* spp. and one each of *Flavobacterium*, *Vibrio* and *Burkholderia*.

Biomass build- up with different carbon sources: Since a limited quantity of tech-HCH can be used as substrate, the biomass build up is low and not sufficient for use. Hence, it is imperative to use other easily utilizable carbon sources as co-substrates for the preparation of inoculum without loosing the HCH-degrading ability. Many simple and complex carbohydrates at levels equal to 1% carbon (equivalent of acetone) along with 25 ppm of HCH, were inoculated with HCH degrading consortium, after 72 h incubation on shaker at ambient temperature. It was found that molasses supported maximum biomass yield followed by glucose and sucrose (Table 1). Among the hydroly-

Table 1. Biomass yield of HCH-degrading consortium after 72 h growth on different carbon sources

Carbon source	Biomass yield, µg protein/ml
Inoculum	23-26
Acetate	160
Succinate	170
Glucose	384
Sucrose	380
Molasses	466
Corn steep liquor	78
Rice straw extract	270
Rice straw extract+glucose	348
Rice straw hydrolysate	314
Corn cob hydrolysate	56
Wheat bran hydrolysate	235
Bagasse hydrolysate	179
Nutrient broth	290

sates and extracts, extract from rice straw supplemented with glucose supported maximum biomass production followed by rice straw and wheat bran hydrolysates. Corn-cob hydrolysate gave lowest biomass production.

The carbon sources, which supported good biomass production, did not show maximum degradation. The consortium grown on wheat bran hydrolysate and HCH caused maximum degradation as only 11% of α-isomer, 5% of γ-isomer, 17% of β- and δ-isomers were found to be present after 72 h incubation (Table 2). Next was molasses which showed nearly 70% degradation. This was followed by succinate. Simple substrates like acetate, glucose and sucrose did not show good degradation. Lowest degradation was observed in rice straw extract supplemented with glucose where nearly 80% of the added substrate could be recovered after 72 h. The low rate of degradation in molasses as compared to wheat bran hydrolysate may be due to low level or absence of induction of essential enzymes of the degradative pathway. The same is true with other substrates where the degradation of tech-HCH was low. It was observed that in molasses, although the biomass build up was highest, all the isolates could not be recovered after 72 h (Table 3), probably these isolates could not utilize molasses as source of carbon and energy. However, in wheat bran hydrolysate, the degradation was maximum and all the 10 isolates of the tech-HCH degrading consortium were recovered after 72 h of incubation. In succinate and acetate although all the isolates of the consortium could be recovered but their number was less.

Pseudomonas fluorescens T_1, was dominant member after 72 h in these substrates, which could have been the reason for low rate of degradation. Consortium grown in glucose and sucrose medium, although supported growth of all members of the consortium but degradation rate was low. The enzymes of the HCH-biodegrading pathway probably are not induced in presence of glucose and sucrose. Vali et al (1992) have reported induction of HCH and DDT degrading enzymes in white rot fungus in presence of glucose.

Thus, suitable substrate is necessary which at optimum concentration would facilitate all the individual members of the consortium to grow without loosing HCH-degrading ability. The individual members appear to act synergistically in degrading HCH. All the individual members of the consortium were able to grow in wheat bran hydrolysate without loosing HCH-degrading ability.

Effect of sugar concentration on degradation of tech-HCH: The wheat bran hydrolysate and molasses grown inoculum, which showed better degradation of HCH were chosen for further studies. The HCH-degrading consortia grown on different concentrations of wheat bran hy-

Table 2. Degradation of HCH by the consortium after 72 h in presence of different co-substrates

Co-substrates	Inoculum biomass, µg/ml	Biomass produced, µg/ml	Chloride released, %	Residual isomers, %			
				α	β	γ	δ
Acetate	30	29.3	54.7	43.3	35.7	28.4	27.4
Succinate	30	20.2	50.9	34.4	32.7	19.7	22.0
Glucose	20	34.4	62.8	46.4	42.7	50.0	30.0
Sucrose	20	47.1	40.3	51.4	51.3	50.0	55.7
Molasses	20	56.1	41.2	29.8	37.8	35.4	42.5
Corn steep liquor	20	11.6	44.3	40.3	46.1	30.6	39.3
Rice straw extract	20	31.2	33.4	74.6	66.6	67.2	76.5
Rice straw extract +glucose	20	40.8	30.4	74.4	66.3	66.1	76.5
Rice straw hydrolysate	20	36.5	53.6	55.4	41.1	46.7	64.6
Corn cob hydrolysate	20	7.2	38.6	40.3	46.1	30.6	39.4
Wheat bran hydrolysate	30	26.8	56.5	11.1	17.1	5.2	17.2
Bagasse hydrolysate	20	2.9	45.4	67.6	69.9	56.0	88.1
Nutrient broth	20	25.6	21.1	30.9	30.3	33.3	25.2

Table 3. Bacterial counts of the consortium grown on different carbon sources containing 25 µg/ml HCH

Carbon source	*Ps. fluorescens** (T1)	*Ps. diminuta* (T2)	*Ps. fluorescens* (T3)	*Burkholderia pseudomallei* (T4)	*Ps. putida* (T5)	*Flavo bacterium* (T6)	*Vibrio alginolyticus* (T7)	*Ps. aeruginosa* (T8)	*Ps. stutzeri* (T9)	*Ps. fluorescens* (T10)
Initial	1.6	1.7	2.0	1.6	1.6	1.6	1.6	2.0	1.7	2.0
Acetate	10.3	6.2	8.9	6.3	6.1	16.3	7.3	6.8	9.6	10.3
Succinate	10.9	10.9	9.7	10.0	8.0	16.4	16.3	8.5	10.9	9.3
Glucose	14.6	12.4	12.0	13.0	12.3	12.3	14.7	13.1	12.0	9.5
Sucrose	14.3	11.5	13.5	11.3	10.4	13.3	8.0	13.9	14.1	14.3
Molasses	13.2	0.0	14.2	14.4	12.9	13.4	14.2	0.0	0.0	0.0
Corn steep liquor	12.6	12.4	12.4	12.0	12.3	12.0	13.8	12.1	13.7	14.4
Bagasse hydrolysate	13.0	14.9	12.3	8.3	11.6	12.0	13.8	14.8	8.0	14.9
Corn cob hydrolysate	12.0	15.3	12.4	12.0	0.0	12.1	0.0	0.0	0.0	0.0
Wheat bran hydrolysate	11.3	6.4	10.9	10.4	11.6	8.4	8.8	11.9	12.8	10.1
Rice straw hydrolysate (RSH)	8.9	3.8	2.0	12.0	8.7	0.0	0.0	0.0	7.6	6.5
RSH+glucose	14.0	0.0	13.3	14.3	9.3	13.4	13.9	0.0	0.0	0.0
Rice straw extract	8.0	0.0	8.0	8.3	8.0	12.3	8.4	0.0	0.0	0.0
Nutrient broth	16.3	17.6	18.0	17.8	17.9	17.9	17.2	17.8	17.8	16.9

Table 4. Degradation of HCH (25 ppm) using inoculum grown on wheat bran hydrolysate and molasses containing 0.5-5% reducing sugars

Reducing sugar, %	Growth µg protein/ml	Cl-released, %	Residual tech-HCH, %*			
			α	β	γ	δ
Wheat bran hydrolysate						
0.5	21	61	26	21	24.7	22
1.0	25	53	19	19	15	11
1.5	26	56	17	77	5	17
2.0	28	53	8	6	6	10
4.0	31	48	7	6	10	94
5.0	36	57	4	5	100	2
Molasses						
0.5	36	33	31	39	36	40
1.0	40	44	31	33	32	42
1.5	32	41	29	37	35	42
2.0	36	41	30	39	38	39
3.0	41	53	31	36	36	44
4.0	44	47	29	40	36	44

*The concentration of individual isomers at 0 h was taken as 100%.
Initial inoculum added was 20 mg protein/ml
Values observed after 72 h

drolysate or molasses containing 0.5 to 5% of reducing sugar, along with 25 ppm of HCH were harvested after 72 h of growth, washed well and inoculated to flasks containing 25 ppm of HCH in mineral medium. Growth (protein), chloride released and residual HCH were analyzed after 72 h of growth. It was observed that with increase in sugar concentration, the increase in growth was only marginal (Table 4). But degradation of HCH improved with inoculum grown with increased concentration of sugar in the case of WBH. In the case of molasses grown inoculum, there was no significant difference.

Thus the tech-HCH degrading consortium developed by mixing α-, β-, γ-, δ- HCH degrading consortia, could degrade tech-HCH very efficiently. High biomass yield was obtained with molasses, sucrose and glucose. But better degrading ability was shown by the inoculum grown on wheat bran hydrolysate. This consortium may prove effective in bioremediating HCH contaminated soils and other contaminated bodies.

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