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Replacement of Hexachlorocyclohexane to Environmentally Friendly Biosurfactant as Precursor for the Production of Biosurfactant from *Pseudomonas*

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Production of biosurfactant can be substantially increased by the addition of precursors like vegetable oils, petroleum products, and other water-insoluble substances. *Pseudomonas* Ptm+ strain produces biosurfactant in the presence of hexachlorocyclohexane (HCH), which specifically emulsifies HCH, a recalcitrant organochlorine pesticide. Addition of previously produced crude biosurfactant by the same organism as a precursor instead of HCH increased production of biosurfactants with a decrease in the total fermentation time from 32 to 24 h. The main objective of this paper was to find alternatives for HCH as an inducer.

Keywords: Biosurfactant, precursor molecule, *Pseudomonas*, xenobiotic, HCH, restrictive medium

Biosurfactants are increasingly becoming important because of their properties such as low toxicity, biodegradable character, and effectiveness at extreme temperature and pH values. Therefore, there is a growing interest in considering biosurfactants as potential alternatives to compounds obtained by chemical synthesis [4]. Addition of surfactants or biosurfactants or both together increased the recovery and biodegradability of hydrocarbons and other toxic organic compounds [2, 9, 12].

Pseudomonas Ptm+ strain has been isolated earlier at CFTRI and it produced a bioemulsifier specific to hexachlorocyclohexane (HCH), a recalcitrant organochlorine pesticide [2]. Production of biosurfactants can be enhanced by growing the organisms on hydrocarbon or vegetable oil [8]. Earlier, it was noticed that addition of HCH increased the synthesis of biosurfactant by *Pseudomonas* Ptm+ strain [2]. As HCH is toxic, downstream processing and cleaning of fermentors require a large quantity of solvents, both of which require further treatment before disposal. Hence, alternate precursors for the synthesis of biosurfactants were explored. This study reports the incorporation of a biosurfactant in the growth medium as a precursor for the stimulation of growth and enhanced biosurfactant production.

Pseudomonas Ptm+ strain was maintained on Seubert's mineral medium [10] consisting of 20 ppm alpha (α) HCH, 5 g of mannitol, and 2.5 g of yeast extract in one liter of distilled water.

Ten percent of the 24-h-old culture was inoculated to Luria broth and was grown for 10 h on a magnetic stirrer (150 rpm) at ambient conditions.

The above inoculum was inoculated at 2.5% to restrictive medium (RM) containing 7 g of K_2HPO_4, 0.1 g of $MgSO_4$, 0.455 g of urea, and 20 g of mannitol in one liter of distilled water, at a pH of 7.2. The above medium was spiked with 100 mg/l of alpha HCH at the time of inoculation, and the culture was grown on a rotary shaker at 200 rpm and 30°C for 30 h. The culture broth was centrifuged at 12,000 ×*g* for 15 min.

The supernatant was collected and extracted three times with petroleum ether (40–60 fractions). Ether fractions were pooled and evaporated under vacuum. Thereafter, the ether-extracted biosurfactant was weighed and dissolved in 0.1N $NaHCO_3$ and used as the precursor.

Biosurfactant dissolved in 0.1N $NaHCO_3$ was used as the precursor in varying proportions [0.25%, 0.5%, and 1.0% (w/v)] for the production of biosurfactant. RM was used as the basal medium. The four combinations tried were HCH (100 mg/l), biosurfactant, HCH and biosurfactant, and RM without mannitol (carbon source) with biosurfactant. The culture was grown on a rotary shaker at 200 rpm and 30°C for 30 h. The culture broth was centrifuged at 12,000 ×*g* for 15 min. The supernatant was used for biosurfactant assay. Biosurfactant assay was carried out as described by Anu Appaiah and Karanth [2].

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Table 1. Effects of biosurfactants and alpha HCH on the growth and emulsifier activity in *Pseudomonas* Ptm+ strain.

Medium	Biosurfactant concentration (%)	Emulsifier activity		Growth peak	
		(h)	OD at 660 nm	(h)	OD at 600 nm
R M + HCH	-	32	0.854±0.05	32	1.330±0.15
R M + HCH+BS	0.25	40	1.226±0.18	22	1.805±0.25
	0.50	24	1.179±0.07	38	1.925±0.18
	1.00	28	0.826±0.5	28	1.610±0.35
RM+BS	0.25	22	0.989±0.25	40	1.610±0.35
	0.50	24	1.087±0.05	48	1.335±0.12
	1.00	26	0.686±0.19	48	1.635±0.24
RM-Man+HCH+BS	0.25	40	0.346±0.07	32	0.900±0.29
	0.50	44	0.359±0.05	32	0.940±0.18
	1.00	24	0.325±0.12	32	0.945±0.32

RM = Restrictive Medium; BS = Biosurfactant; HCH = alpha HCH; Man = Mannitol. Biosurfactant concentration was calculated as % (w/v). Cell growth was measured turbidimetrically at 600 nm against distilled water blank. Emulsifier activity was determined as described by Anu Appaiah and Karanth [2]. Units: 1 unit is defined as the change of absorbance by 0.1 at 660 nm under the assay conditions.

In our earlier report where culture was grown in RM containing alpha HCH, maximum growth and biosurfactant production were observed at 32 h [2]. It can be seen from Table 1 that for each of the different combinations, the growth peak and the biosurfactant peak differed. On comparison of the trials where the culture was spiked with biosurfactant as a precursor, maximum surfactant activity was achieved with 0.5% spiking. The action of biosurfactant as a precursor was independent irrespective of whether alpha HCH was added or not. Similar experiment in the absence of alpha HCH indicated that HCH was not required by *Pseudomonas* for the synthesis of biosurfactant [6]. Experiments where biosurfactant was used as sources of carbon (RM without mannitol) produced low growth and bioemulsifier (Table 1).

Kinetics of bioemulsifier production indicated that when biosurfactant was used as a precursor, maximum emulsifier activity was observed (1.0 unit) (Fig. 1) as compared with HCH as the precursor (0.854 unit). Kinetics of synthesis indicated that there were two peaks of surfactant activity when biosurfactant was added to the media, irrespective of the presence or absence of HCH. When alpha HCH was used along with biosurfactant, a maximum of 1.179 biosurfactant units of activity was observed at 24 h as compared with the peak maxima at 32 h when only HCH was added. This implies that the presence of biosurfactant in the medium induces higher and earlier production of biosurfactant. This could be due to solubilization and mobilization of the alpha HCH present in the culture medium. Surfactants make insoluble substances more readily accessible to the cells [9]. Another possible explanation would be that the biosurfactant present in the medium induces production of specific enzymes, which assist in degradation of biosurfactant added to the media, so that the

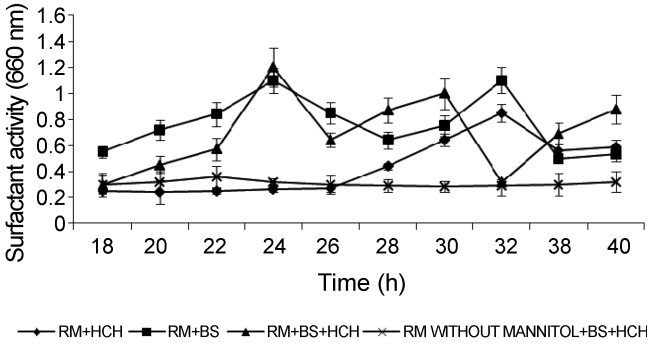

Fig. 1. Kinetics of bioemulsifier production by *Pseudomonas* Ptm+ strain [RM = Restrictive Medium; BS = Biosurfactant (0.5%); HCH = 100 ppm alpha HCH; RM-Man = Restrictive Medium without mannitol].

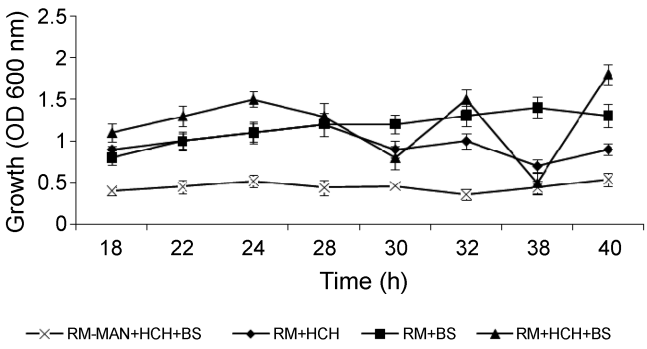

Fig. 2. Growth of *Pseudomonas* Ptm+ strain during biosurfactant production (RM = Restrictive Medium; BS = Biosurfactant; HCH = alpha HCH; RM-Man = Restrictive Medium without mannitol).

resulting substitute lipid is made easily available to the cell to produce biosurfactant (salvage pathway). Boulton and Ratledge [5] and Karanth and Anu Appaiah [6] have emphasized the requirement of a lipid moiety for the synthesis of biosurfactant by *Pseudomonas*. This theory explains the fluctuation in emulsifier activity occurring during the time course (Fig. 1). It is not certain if this is a prerequisite for surfactant production, as some biosurfactants are produced in abundance even when water-soluble substrates are used [7]. Earlier, Sotirova *et al*. [11] indicated that addition of biosurfactant provokes a multicomponent response of *Pseudomonas*. This response is dose-dependent with low concentrations of biosurfactant having no effect on the lipopolysaccharide (LPS) component of the bacterial outer membrane, whereas higher concentrations reduce the LPS content by 22%. In the present work, addition of bioemulsifier above 0.5% as the precursor decreased the production of bioemulsifier.

Growth maxima of the culture were delayed when bioemulsifier was added as the precursor and it took 32 h with HCH (with or without mannitol), 38 h in combinations of HCH and bioemulsifier, and 38 h with biosurfactant alone (Table 1). The combinations of HCH and bioemulsifier indicated diauxic growth pattern, indicating the utilization of various carbon sources (mannitol, biosurfactant, and HCH) at different stages of growth (Fig. 2). Such diauxic growth in tandum with bioemulsifier production was earlier reported by Anu Appaiah and Karanth [3], who had observed the synthesis of lipase as a requirement for the acceleration of growth. Maximum growth was observed in combinations with HCH and biosurfactant. The time taken for maximum growth increased as the concentration of bioemulsifier increased, irrespective of the addition of HCH (Table 1). Earlier, we had observed that mannitol was the preferred sugar for the synthesis of biosurfactant by *Pseudomonas* [1]. However, the present result indicates the requirement of sugar for the biomass production and emulsifier synthesis. In the absence of mannitol, the growth and bioemulsifier production were low (Fig. 1 and 2).

In conclusion, this is the first confirmatory report that the presence of biosurfactants produced by the same organism in the medium aids in the growth and surfactant production. The mechanism(s) by which biosurfactants stimulates the growth of the microorganism is yet to be understood. However, it is believed that biosurfactant increases the cell hydrophobicity by decreasing the major outer membrane protein contents [11]. This study proposes future use of the biosurfactant itself as the precursor molecule instead of the recalcitrant xenophobic molecules.

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