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Factors influencing polyhydroxyalkanoate accumulation in marine bacteria

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Six bacterial isolates from marine ecosystem accumulating > 1 g polyhydroxyalkanoates (PHA) per liter culture broth were characterized and identified. Various physicochemical parameters such as time, aeration, *p*H, carbon and nitrogen content, carbon substrates and organic nitrogen sources were optimized for PHA production by these selected cultures. The PHA yield from these cultures on optimization ranged between 1.4 gl^{-1} to 3.9 gl^{-1} culture broth.

[**Key words:** Physicochemical parameters, polyhydroxyalkanoates, bacterial species.]

[**IPC Code:** Int. Cl.[7] C12 Q 1/02, C 12P 1/04, C 12P 39/00]

Introduction

Polyhydroxyalkanoates (PHA) are biodegradable polyesters synthesized as high-energy carbon reserve by microbes and have tremendous potential as substitutes for petrochemical plastics[1]. Bacteria accumulating PHA have been reported from various environments like soil, sewage sludge, ponds and tropical mangrove and marine environments[2-4]. Use of a high PHA yielding organism, optimization of the physico-chemical parameters[5,6] and reduction of the cost of the raw materials are expected to lower the prohibitively high cost of PHA production. Present work shows that the yield of PHA from the bacteria can be increased effectively by optimization of various physicochemical parameters.

Materials and Methods

Several bacteria accumulating PHA were isolated from the tropical mangrove and marine water and sediment samples from the Bombay high oil fields and coast of Goa and Karwar[4]. The PHA extracted from the six cultures designated as 61/4, 64/4, 87/4, 12/BL, 85/6 and 86/6 were found to be homopolymers of hydroxybutyrate, exhibiting varied physical and mechanical properties[7]. These six isolates accumulated more than one gram of PHA per liter of culture broth and were used in the present work. The cultures were identified to generic level using routine microscopic, cultural and biochemical analysis, based on the Bergey's manual of systematic bacteriology[8, 9].

The cultures were grown in 50 ml of E2 mineral medium[10] and supplemented with 0.04% (w/v) yeast extract, 2% (w/v) glucose in Ehrlenmeyer flasks, incubated at 28°C on an Orbitek environmental shaker. Time dependent studies were carried out by withdrawal of a pair of flasks at each time interval (4 h). The growth was monitored turbidimetrically at 420 nm and the changes in *p*H were noted.

Effect of varying concentration of glucose and microcosmic salt [$(NaNH_4H)PO_4$] was checked on PHA accumulation in E2 mineral medium. Effect of varying carbon substrate was checked by substituting glucose with 2% (w/v) of carbohydrates like sucrose, fructose, lactose, maltose, mannitol or with 1% (w/v) sodium benzoate and sodium acetate. Effect of substituting yeast extract with other complex nitrogen sources such as beef extract, casein, tryptone, meat extract, peptone and malt extract in the concentration 0.04% (w/v) was also studied. The observations presented are the means of three independent experiments.

PHA accumulated in the bacterial cells were extracted by the modified hypochlorite method[11]. The bacterial cells were grown in mineral medium at 28°C on an Orbitek environmental shaker for 48 h. The cell pellet from 10 ml culture, washed in saline was suspended in 10 ml of sodium hypochlorite with 2% (w/v) active chlorine and incubated for 10 min at

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37°C with constant shaking. Pellet of PHA obtained on centrifugation at 8000 rpm for 20 min, was washed with 10 ml of cold diethyl ether and assayed with concentrated sulphuric acid[12]. PHA sample (0.5 ml) was treated with 4.5 ml of concentrated sulphuric acid in a boiling water bath for 10 min. The contents were mixed thoroughly, but gently. The absorbance was determined at 235 nm against a blank, distilled water, treated in the same manner as the PHA samples. Standard (DL)-β-hydroxybutyric acid (Sigma chemical) in the concentration range 50-300 nM was used as the standard.

Production of PHA by the culture 85/6 was scaled up to three liter level using a five liter bioreactor (BIOSTAT Braun Biotech international). The impeller speed was set at 300 rpm, aeration rate at 1 gl^{-1} and the temperature was maintained at 37°C. Sampling (5 ml) was done every 4 h and analysed for PHA and growth.

Results and Discussion

The taxonomically distinctive biochemical characteristics of the six isolates selected for the studies on PHA accumulation are enumerated in Table 1. Each of the isolates selected for present study were non-sporulating rods, 61/4, 64/4 and 87/4 isolated from seawater being Gram positive, 12/BL from mangrove sediments and 85/6 and 86/6 from marine sediments being Gram negative. The isolate 12/BL produced a violet pigment, showed fermentative metabolism of glucose, lecithinase and caseinase activity, produced HCN and was identified as *Chromobacterium violaceum*. Cultures 85/6 and 86/6 require Na^+ for growth, hydrolyse arginine and possess distinct polar flagellum, utilize sodium benzoate as sole carbon source and were classified as *Vibrio nereis*. Culture 87/4, pleomorphic rods, possess mycolic acids and m-diaminopimelic acid in their cell walls and were identified as *Corynebacterium* spp, while the cultures 61/4 and 64/4, regular motile rods require organic growth factors, lack mycolic acids in cell walls and therefore were classified as *Listeria* spp.

Accumulation of PHA has been reported in *Corynebacterium* sps.[13], *Vibrio parahaemolyticus* and *V. harveyi*[14] and *Chromobacterium violaceum*[15]. Production and accumulation of PHA by *Listeria* spp. have not been hitherto reported. The accumulation of PHA by the *Listeria* sp. 61/4 and 64/4, reflects upon the diversity and the potential of the tropical coastal ecosystem to harbour wider range of organisms accumulating PHA.

Table 1—Culture and biochemical characteristics of the selected isolates

Isolates	61/4	64/4	87/4	12/BL	85/6	86/6
Staining						
Gram's	+	+	+	-	-	-
Polar flagella	-	-	-	+	+	+
Morphology	RR	RR	IR	SR	SR	SR
Motility	+	+	-	+	+	+
Oxidase	-	-	+	+	+	+
O-F test	F	F	F	F	F	F
Catalase	+	+	+	+	+	+
NO^{3-} to NO^{2-}	+	+	-	+	+	+
Required for growth						
Na^+	-	-	-	-	+	+
Growth factor	+	+	+	-	-	-
Lipase	+	+	+	-	-	-
Amylase	+	+	ND	-	ND	ND
Gelatinase	+	+	ND	+	ND	ND
Cellulase	+	+	+	-	-	-
Arginine hydrolysis	-	-	-	+	+	+
Genus	*Listeria*	*Listeria*	*Coryne-bacterium*	*Chromo-bacterium*	*Vibrio*	***Vibrio***

Key: + = positive; - = negative reactions; ND = Not done; O-F = Oxidative-Fermentative glucose metabolism by Hugh and Leifson's method; RR = regular rods; IR = irregular rods; SR = short rods.

The accumulation of PHA begins at the late log phase, reaching maximum in the stationary phase of growth (Fig. 1), followed by fall during further incubation for most of the isolates except culture 12/BL and 85/6 for which the PHA yield plateaued, a characteristic useful in large scale production of PHA.

Aeration and *p*H of the growth medium are two important physical parameters that influence growth and accumulation of PHA. Though the lower aeration rates favour PHA accumulation, it doesn't seem to be true for all cultures[6]. Maximum PHA was accumulated by *Vibrio* spp. 85/6 and 86/6 at aeration levels of 200 rpm whereas isolates 87/4 and 12/BL at 100 rpm and isolates 61/4 and 64/4 at 150 rpm. All the isolates showed the optimum accumulation of PHA when grown in medium at *p*H 7.0 (Table 2). Medium at *p*H below 6 did not support polymer accumulation, although the onset of PHA

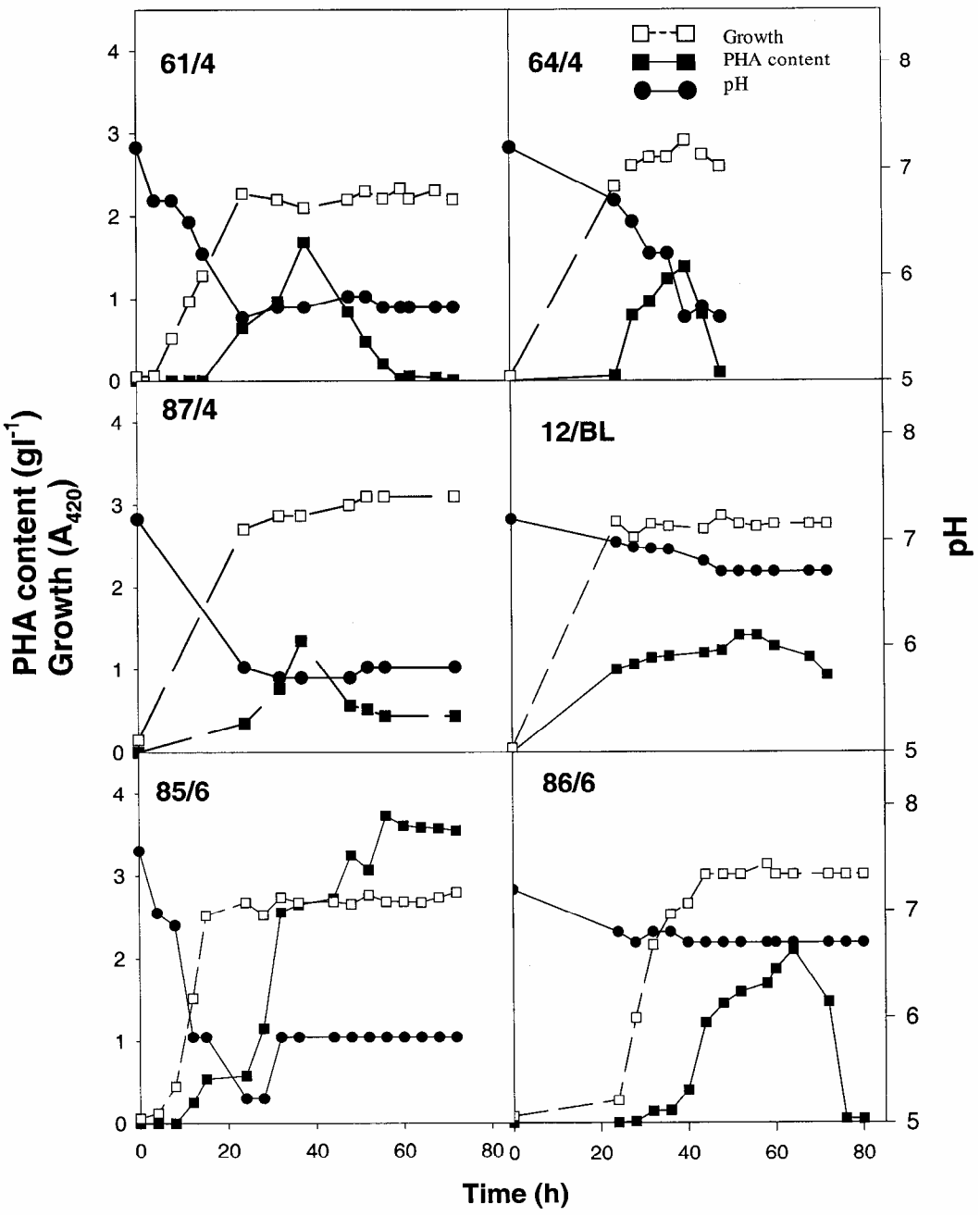

Fig. 1—Accumulation of PHA in the selected six isolates during growth in the E2 medium.

accumulation coincides in most cultures with the slight drop in *p*H of the medium during the growth (Fig. 1).

With an increase in the glucose concentration, PHA accumulation was seen to increase till a threshold, after which it either remained steady or declined (Fig. 2A). Under reduced concentrations of nitrogen and phosphorus, PHA accumulation was reported to improve[5, 16-19]. A significant increase, in the amount of PHA is seen in *Vibrio* sp. 85/6 by limiting the nitrogen content (Fig. 2B).

For all the cultures, glucose was substrate of choice, giving the highest yield of PHA, followed by sucrose (Fig. 3A). *Vibrio* sp. 85/6 is the only isolate

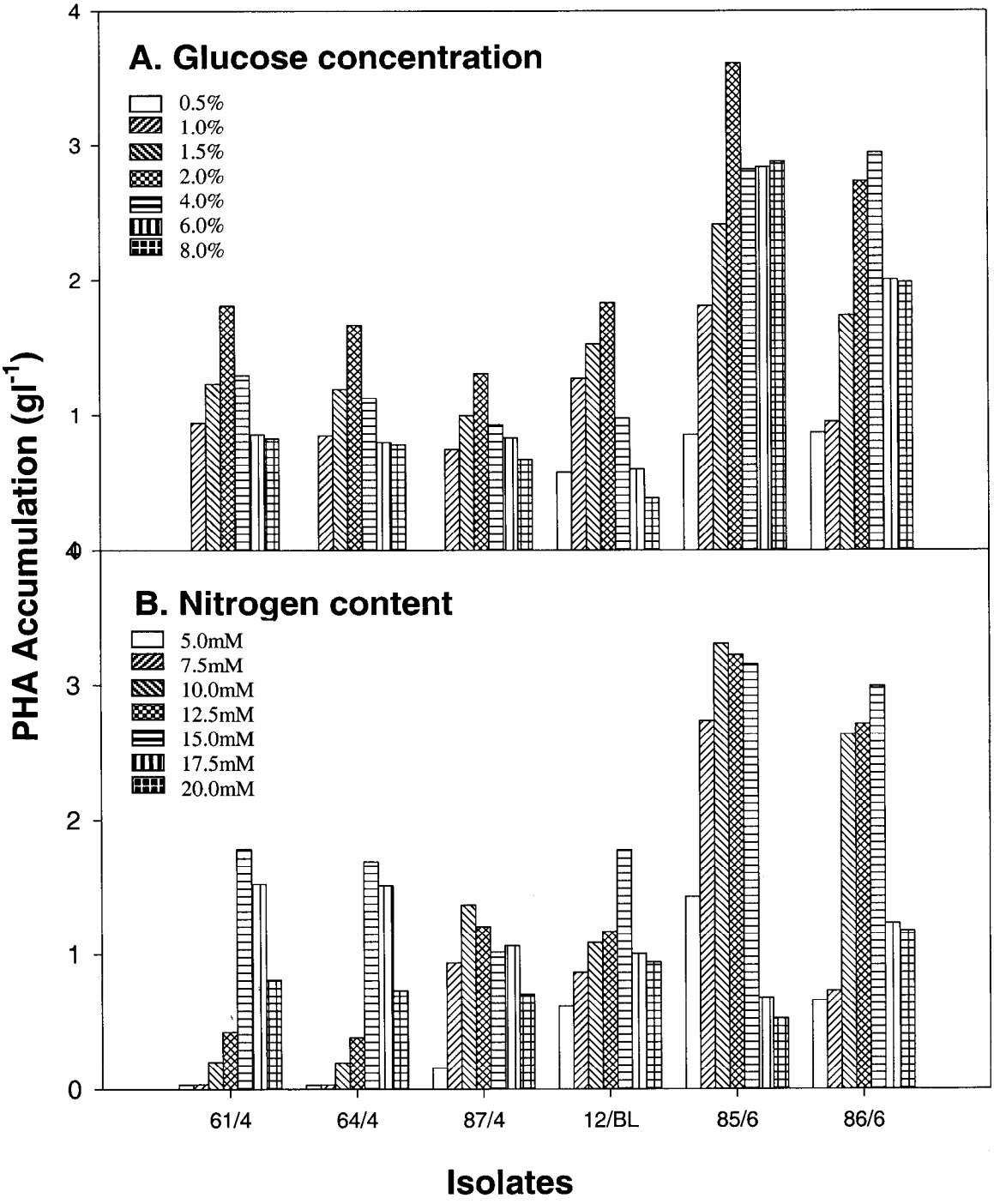

Fig. 2—Influence of concentration of (A) glucose and (B) microcosmic salt-nitrogen content on PHA accumulation in the selected six isolates.

yielding PHA from maltose and significantly high PHA in fructose. The Gram negative cultures seem to be more versatile in utilizing various carbon sources; both *Vibrio* spp. could utilize aromatic hydrocarbons and *C. violaceum* and *Vibrio* spp. could utilize acetate for PHA accumulation (Fig. 3A). Such cultures could be made to use cheaper raw materials, agricultural and industrial wastes[1-3], thus offering a higher potential for economic production of PHA. Maximum accumulation of PHA by each of the isolates was lowered considerably when yeast extract was substituted with malt extract (Fig. 3B).

It is seen that each of the six cultures showed a different response towards the various physicochemical parameters (Table 2). As high as three fold increase in the yield of the polymer was

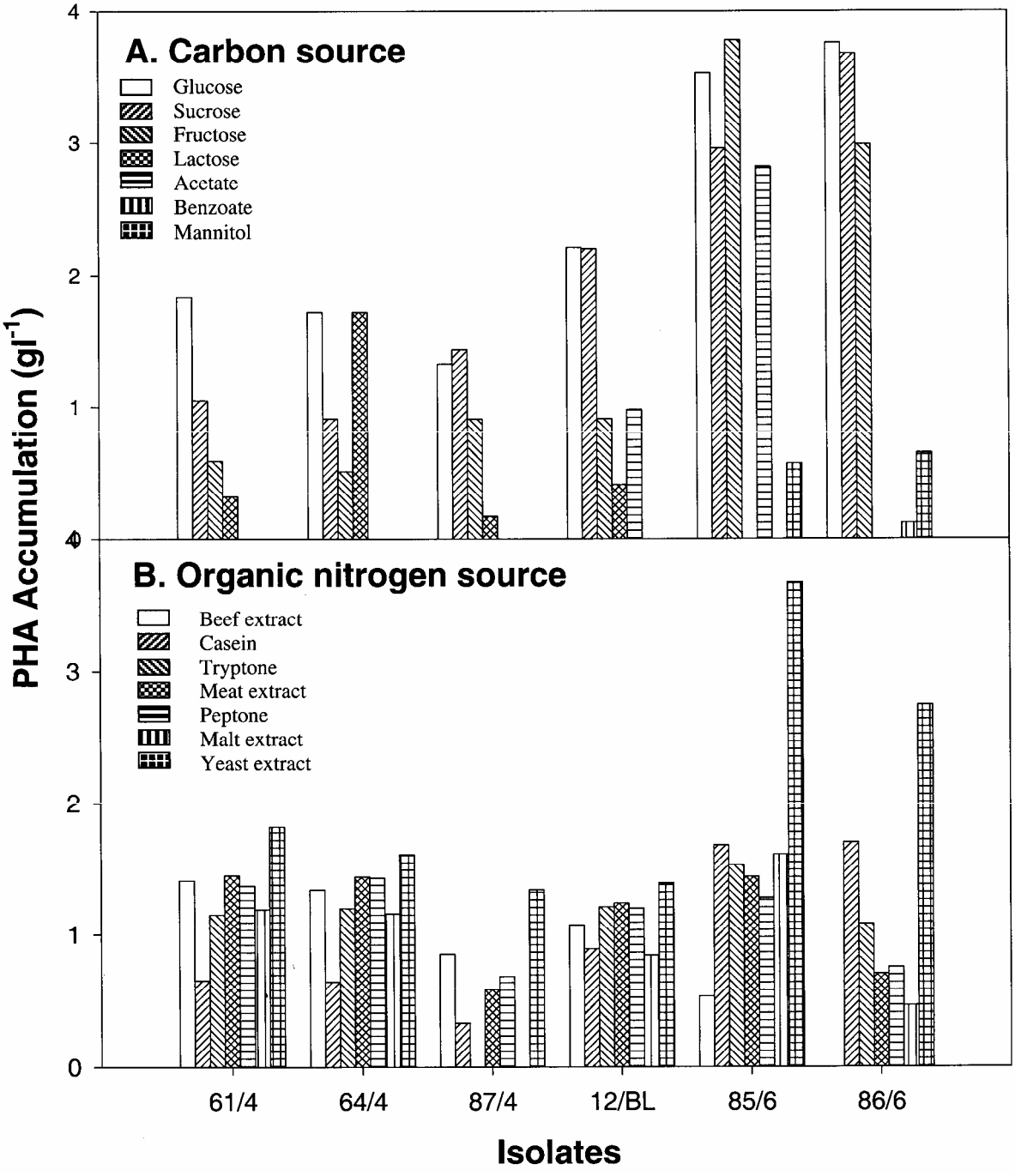

Fig. 3—Influence of (A) carbon substrate (B) organic nitrogen source on PHA accumulation in the selected six isolates.

Table 2—Optimized conditions for maximum yield of PHA in the selected bacterial isolates during growth in E2 medium

Isolates	61/4	64/4	87/4	12/BL	85/6	86/6
Time (h)	38	38	38	56	56	64
*p*H	7.2	7.2	7.2	7.2	7.2	7.2
Aeration (rpm)	150	150	100	100	200	200
Glucose (%w/v)	2	2	2	2	2	4
Nitrogen (mM)	15	15	10	15	10	15
Organic GF	YE	YE	YE	YE	YE	YE
Carbon source	Gl	Gl	Gl	Gl/ Su	Fr	Gl
PHA yield (gl^{-1})	1.8	1.7	1.4	2.2	3.9	3.7

GF = Growth factor; YE = yeast extract; Gl = glucose; Su = sucrose; Fr = fructose.

obtained by optimization of some of the parameters in the culture *Vibrio* sp. 85/6. The behaviour of this culture in the five-liter fermenter in terms of the accumulation of polymer (3.9 gl^{-1}) and the optimum time (56 h) coincides with that of the shake flask. Optimization, using such parameters, not only increases the polymer yield, but also helps in reducing the cost of the polymer and may produce a polymer with desirable properties.

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