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Fermentation optimization for the production of poly(β-hydroxybutyric acid) microbial thermoplastic

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Abstract

Batch culture of *Alcaligenes latus*, American Type Culture Collection 29713, was investigated for producing the intracellular bioplastic poly(β-hydroxybutyric acid) (PHB). A central, composite experimental design was used to optimize the composition of the culture medium for maximizing the productivity of PHB. Investigated were the effects of temperature, the initial culture pH, the ionic strength of the medium, the concentration of trace elements, the type of nitrogen source, and the carbon-to-nitrogen ratio. The optimal temperature for growth and PHB synthesis appeared to be 33°C; however, over the 25–37°C range, the effect of temperature was negligible. An initial pH value of 6.5 gave the best results; pH values that differed even slightly from the optimum reduced the culture performance. Typical culture characteristics were: 0.075/h maximum specific growth rate, 0.38 g/l h maximum specific sucrose consumption rate, and 0.15 g/l h maximum specific PHB production rate. PHB was lost because of hydrolysis in the stationary phase, suggesting critical importance of timing the harvest. Under the best conditions, PHB constituted up to 63% of dry cell mass after 93 h of culture. The average biomass yield coefficient on sucrose was about 0.4 kg/kg. Of the four nitrogen sources—ammonium chloride, ammonium sulfate, ammonium nitrate, and urea—used, only the first two supported the culture satisfactorily. The biomass and PHB showed clear yield maxima at 1.5 g/l ammonium chloride (C:N ratio = 21.5) and 1.4 g/l ammonium sulfate (C:N ratio = 28.3). The yields were higher with ammonium sulfate and were relatively more sensitive to changes in its concentration. Ionic strength had a strong negative effect on PHB productivity. The highest PHB yield occurred at 4 g/l phosphate buffer concentration. Iron appeared to have the potential to enhance the proportion of PHB in the cells.

Keywords: Poly(β-hydroxybutyric acid); Polyhydroxybutyrate; Polyhydroxyalkanoate; *Alcaligenes latus*; Biopolymer; Bioplastic

1. Introduction

Poly(β-hydroxybutyric acid) (PHB) is an intracellular microbial thermoplastic that is widely produced by many bacteria [1–3]. In terms of molecular weight, brittleness, stiffness, melting point, and glass transition temperature, the PHB homopolymer is comparable to some of the more common petrochemical-derived thermoplastics, such as polypropylene [4]. Therefore in certain applications, PHB can directly replace some more traditional, nonbiodegradable polymers. Wider use of PHB, primarily as polymer blends, is expected. Such blends will greatly increase the spectrum of possible applications by expanding the range of available physical properties. PHB, in combination with other biocompatible and nontoxic polymers, would also have an enhanced scope in biomedical applications. Because PHB is resistant to water and ultraviolet radiation, and it is impermeable to oxygen, it is especially suited to use as food packaging. PHB is readily biodegraded in soil. Moreover, it can be processed by using the same technology that is currently used in making polyethylene or polypropylene components. PHB has been used in surgical sutures [3], and other uses are in development [5]. The high price of commercial grade PHB—about 15-fold greater than comparable synthetic plastics—limits its use to specialist niches. For example, Biopol®, a copolymer of β-hydroxybutyric acid and β-hydroxyvaleric acid produced by *Ralstonia eutropha*, sells for about 17 times the price of synthetic plastics [3]. Wider use of PHB requires a less expensive product; hence, improved fermentation strategies, low-cost media, and eas-

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ier downstream recovery methods are needed [6–8]. This work reports on the optimization of PHB production in the bacterium *Alcaligenes latus*.

Microbial production of PHB has predominantly relied upon the bacterium *A. eutrophus* [9,10] and, to a lesser degree, *A. latus* [11,12]. *A. eutrophus* accumulates PHB when a nutrient, such as phosphate, limits its growth but the carbon substrate is present in nonlimiting amounts. This necessitates a more expensive two-stage cultivation: first, the cells must be grown to a high concentration under nonlimiting conditions, followed by a PHB-accumulating second phase. *A. latus*, on the other hand, is a growth-associated producer of PHB; hence, a single-stage fermentation is sufficient [11,13]. Furthermore, *A. latus* can be cultured readily on sucrose, which is less expensive than the glucose that is typically used in *A. eutrophus* fermentations. Up to 80% of dry cell mass of *A. latus* can be PHB. Because of these fundamental advantages, the fermentation optimization studies reported here focus on *A. latus*.

A. latus is a nonsporulating, Gram-negative, obligate aerobe. The bacterial cells are short rods or coccoids, 1.2–2.4 μm in diameter and 1.6–2.4 μm in length. The cells occur singly, in pairs, or in short chains [14,15]. The bacterium is sluggishly motile by means of 5–10 peritrichous flagella [14,15]. Optimal growth temperature is about 35°C. Growth occurs over the pH range 6.0–7.5. Colonies are opaque, yellowish to grayish pink, and round.

As for any fermentation [16,17], the *A. latus* culture performance is affected by numerous variables, including temperature, pH, carbon-to-nitrogen ratio in the feed, concentration of substrates, concentration of trace elements, ionic strength (IS), agitation intensity, and dissolved oxygen. Optimization of fermentation conditions has been used to substantially enhance yield and productivity of many bioprocesses [18,19]. Conventionally, fermentations are optimized empirically by using one-at-a-time variation of process parameters. This approach is inefficient and time consuming. Moreover, it assumes that the various fermentation parameters do not interact and that the process response is a direct function of the single varied parameter. In contrast, the observed behavior of a fermentation results from the interactive influences of the various variables. Unlike conventional optimization, statistical optimization methods can take into account the interaction of variables in generating the process response [20]. Factorial design of optimization experiments is especially suitable to account for the interactions, and it is efficient. A combination of factors (independent process variables) that generate a certain optimum response can be identified through factorial design and the use of response surface methodology [21,22]. A central composite factorial experimental design [20,21] was used for the medium optimization reported here. In this approach, concentrations of medium components are the variables; each variable is referred to some base value and varied in a certain pattern. This pattern was designed by using statistical methods to yield the most information from a minimum number of experiments. As with any statistical experimental design, attention to replication, randomization, and blocking is necessary.

Whereas the biochemistry of PHB synthesis in *A. eutrophus* is known [23], similar information for *A. latus* is not available. Nevertheless, by analogy with *A. eutrophus*, enzymes such as β-ketothiolase A, acetyl-coenzyme A reductase, and PHB synthase are likely to be involved. Moreover, because PHB production in *A. latus* is growth-associated, all enzymes participating in cellular metabolism are likely to affect PHB production, even if indirectly. Some of those enzymes may be sensitive to amounts of trace elements; hence, optimization of trace element concentration in the culture medium was an important aspect of the work reported.

A knowledge of elemental composition of biomass is a useful starting point for optimization of the formulation of the culture medium. A comparison of the elemental composition of *A. latus* [24] with typical ranges for bacteria [25] reveals a general similarity, but there are two notable exceptions: the C:N ratio in *A. latus* (7.7) is higher, and, at 21.5, the C:Fe ratio is substantially lower than the 230–2600 range that is typical for bacteria. The relatively high C:N ratio in *A. latus* is probably explained by the large amounts of PHB in the cells; PHB does not contain nitrogen.

The PHB yield optimization approach demonstrated here nearly doubled the yield by identifying a suitable composition of the culture medium and the environmental conditions through statistically directed experimentation. Unlike conventional developmental methods, the experimental effort was minimal because multiple parameters were varied in a given fermentation batch. This reduced the number of fermentations, time, labor, and consumption of material, while allowing a comprehensive investigation of the culture behavior. A systematic optimization approach has not been applied to *A. latus* before. The results reveal important effects of the C:N ratio, the type of the nitrogen source, and the IS on productivity of PHB.

2. Materials and methods

2.1. Microorganism and culture conditions

An intracellular PHB producer, *A. latus*, ATCC 29714 (or DSM 1123), was used throughout. The strain was maintained on agar slants and Petri dishes on the minimal medium 1018 as noted in the American Type Culture Collection catalog [26]. After sufficient growth at 33°C, the culture was held at 4°C until needed. Shake flasks (200 ml) containing 50 ml of minimal medium 1018 were inoculated with a loopful of cells. After 3–4 days growth (33°C, 200 rev./min shaker), the 50 ml of inoculum attained cloudiness. The inoculum was used directly, or held at 4°C until needed. Shake flask optimization experiments used 1 ml of inocu-

lum for 200 ml working volume in 500-ml flasks. All media were sterilized at 121°C, 20 min, and cooled to 33°C prior to use.

2.2. Cell dry mass

Biomass content were evaluated by gravimetry. Culture samples (10 ml) were centrifuged (15 000 × *g*, 4 min, 4°C), the supernatant was refrigerated for further analysis, and the cell pellet was washed in deionized water, recovered (15 000 × *g*, 4 min, 4°C), dried to constant weight (90°C, 24 h), cooled in a desiccator, and weighed. The biomass yield coefficient on sucrose ($Y_{X/S}$) was calculated as the cell dry weight produced per unit mass of sucrose consumed. All measurements were in duplicate.

2.3. Sucrose

The supernatant (1 ml) from biomass determinations was used to quantify sucrose by refractive index measurements in an Abbe refractometer (Atago 3T, Japan). The refractometer had been calibrated by using dilutions of freshly prepared sucrose-containing culture medium. In addition, a few of the measurements were verified by high-performance liquid chromatography (Millipore) on a lead sulfate column. Automatic sample injection (Waters 700 Saterlite WISP, 20 μl injection volume) was employed. An external differential refractometer (model R401) was used as the detector. Chromatograms were acquired and integrated with a Baseline 810 Chromatography Workstation . Double deionized water was the eluent (85°C, 0.6 ml/min). The high performance liquid chromatography calibration standards were identical to those used for the refractometer.

2.4. Ammonium

A calibrated ammonia sensor electrode (model 8002–8, Electrical Instruments, Chertsey, UK) was used to measure the NH_4^+ concentration. The measurement range was 50–2000 mg/l as NH_4^+. Just before measurement, the supernatant (5 ml) from cell dry mass determinations was made alkaline with concentrated sodium hydroxide (1 ml) to convert the dissolved ammonium ion to ammonia.

2.5. PHB

A gravimetric method similar to those employed previously by Marchessault et al. [10] and Ramsay et al. [27] was used. A sodium dodecyl sulfate solution (1% w/v sodium dodecyl sulfate, 10 ml, pH 10) was added to the biomass pellet obtained as described for cell dry mass measurements. The mixture was incubated on an orbital shaker (60 min, 200 rev./min, 37°C). The solids were recovered by centrifugation (4 min, 7000 × *g*) and washed with commercial sodium hypochlorite solution (Javex-5, Colgate–Palmolive; 1 ml, 5.64% w/v sodium hypochlorite) that had been diluted to 20 ml. The pellet was centrifuged (4 min, 7000 × *g*), washed with deionized water (20 ml), and centrifuged again. The final pellet was dried (90°C, 24 h) to constant weight in preweighed aluminum dishes. The PHB yield coefficient relative to biomass ($Y_{P/X}$) was calculated as the mass of PHB obtained per unit cell dry weight. Measurements were in duplicate.

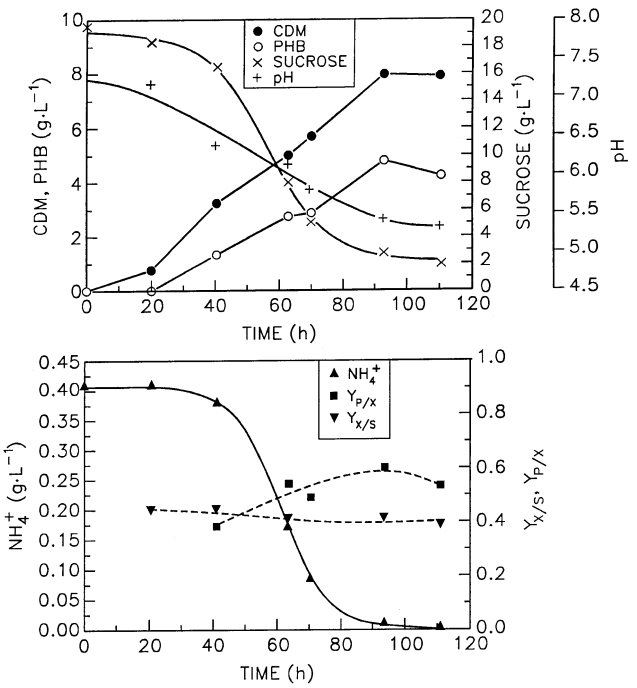

Fig. 1. Typical profile of *A. latus* fermentation. Time variation is shown for biomass (CDM), PHB, sucrose, pH, ammonium, and the calculated values of the yield coefficients $Y_{P/X}$ and $Y_{X/S}$. Data were obtained at 33°C in 500-ml shake flasks.

3. Results and discussion

3.1. Batch culture profile

Industrial fermentations are conducted predominantly as batch and fed-batch operations because long-term, continuous cultures are susceptible to contamination and strain degeneration [19]. Commercial production of PHB also relies on batch and fed-batch culture [11,13,23]; hence, the optimization studies reported here were conducted in those modes, rather than in chemostat continuous culture. Data were obtained in shake flasks (500 ml) with 200 ml working volume (33°C, 200 rev./min). Fig. 1 depicts a typical fermentation profile, the calculated specific product yield coefficient ($Y_{P/X}$), and the growth yield coefficient ($Y_{X/S}$) on sucrose. The culture characteristics were: 0.075/h maximum specific growth rate, 0.38 g/l h maximum specific sucrose consumption rate, and 0.15 g/l h maximum specific PHB production rate. The maximum PHB yield was 60% of the

Table 1
Comparison of culture media

Component	Composition (g/l)			
	Medium 1	Medium 2	Medium 3	Medium 1018
Sucrose	20	20	20	20
$(NH_4)_2SO_4$	2	5.16	1.4	1
KH_2PO_4	1.5	0.14	1.5	4.4
Na_2HPO_4	3.6	0.68	1.8	4.8
$MgSO_4 \cdot 7H_2O$	0.2	0.3	0.2	0.5
Trace element solution (ml/l)	1	1	1	1
	Composition of trace element solution (g/l)			
	TES 1	TES 2	TES 3	TES 4
Ammonium Fe (III) citrate	60	60	6	50
$CaCl_2 \cdot 2H_2O$	10	6.2	10	5
H_3BO_3	0.3	2.8	0.3	—
$CoCl_2 \cdot 6H_2O$	0.2	3	0.2	—
$ZnSO_4 \cdot 7H_2O$	0.1	1	0.1	—
$MnCl_2 \cdot 4H_2O$	0.03	2	0.03	—
$Na_2MoO_4 \cdot 2H_2O$	0.03	0.5	0.03	—
$NiSO_4 \cdot 7H_2O$	0.02	1	0.02	—
$CuSO_4 \cdot 5H_2O$	0.01	0.4	0.01	—

dry cell mass after 93 h of culture. PHB loss because of hydrolysis was observed (Fig. 1) upon reaching the stationary phase, suggesting critical importance of timing the harvest. Because PHB is quite stable in aqueous suspensions [7,8], the observed hydrolysis was associated to enzyme-mediated metabolic action in a low-sugar environment. In most microorganisms, PHB is a food reserve that is degraded to provide carbon and energy when an external carbon source is exhausted.

The specific product yield ($Y_{P/X}$) increased by about 50% during exponential growth, suggesting that cells accumulated PHB while growing and aging; hence, any strategy that prolongs the growth phase is likely to further enhance the PHB yield per unit biomass. The average biomass yield coefficient on sucrose was about 0.4 kg/kg. Less than 2 g/l sucrose remained at the end of fermentation. Both sucrose and nitrogen consumption patterns (Fig. 1) were consistent with a lag-exponential-stationary growth pattern of biomass. Low nitrogen levels toward the end of fermentation may have limited growth. The culture pH was not controlled, and it declined from about 7.4 to 5.2.

A published elemental analysis [24] of *A. latus* was used to establish Medium 2 (Table 1) as the basal medium for optimization experiments. Relative to the other media listed (Table 1), the Medium 2 was high in ammonium sulfate, but low in phosphates. At the other extreme, the Medium 1018 was low in ammonium sulfate, but high in phosphates. The biomass yields obtained in various media are shown in Fig. 2. Medium 3 provided the highest yields. Medium 1018, which was the simplest and the least expensive, was also the least effective. Medium 2, based on the elemental composition of cells, was probably limiting in phosphates. The PHB yield in Media 1–3 averaged 53 ± 1.3% of dry cell mass, whereas the yield in Medium 1018 was higher at 61.5% of cell mass; nevertheless, the total amount of PHB was only about 67% of the best case results in Medium 3. In view of the results of Fig. 2, an optimal production medium needs to provide a satisfactory combination of the cell biomass yield and a high PHB content in the cells.

3.2. Effect of trace elements

To separate the effect of trace elements from those of phosphate buffer and ammonium sulfate, the Medium 1 (Table 1) was supplemented, in separate experiments, with trace element solution (TES) 1–4 (Table 1). The results are shown in Fig. 3, which also includes data in Medium 1 without any added trace elements. As shown in the figure, neither the low trace element media (TES 4, or no trace

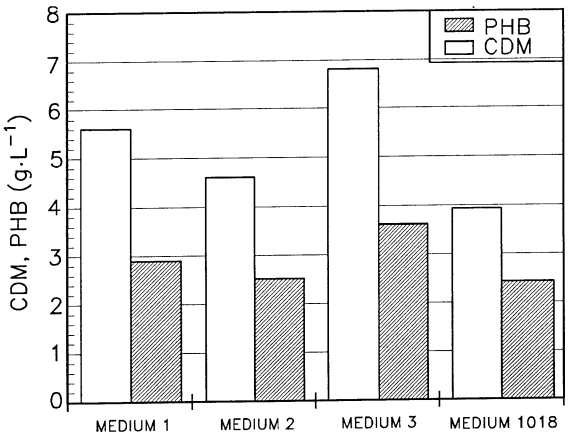

Fig. 2. The biomass (CDM) and PHB yields in various media.

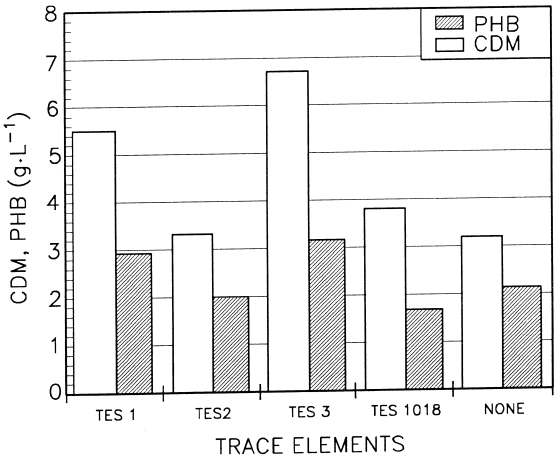

Fig. 3. Effects of various levels of TES supplementation on biomass (CDM) and PHB yields in Medium 1. The TES 1018 corresponds to Medium 1 supplemented with TES 4.

elements) nor that excessively rich in trace elements (TES 2) produce high yields of biomass or PHB. The TES 3 yielded more biomass; however, the overall PHB yields of TES 1 and TES 3 were comparable, even though the latter was low in iron. These results suggest that iron may have the potential to enhance the proportion of PHB in the cells.

3.3. Carbon-to-nitrogen ratio

Ammonium sulfate (0–5.2 g/l) and ammonium chloride (0–4.215 g/l) were used to vary the C:N ratio in separate experiments. Other media components were as listed for Medium 1 in Table 1. The results are shown in Fig. 4. Both biomass and PHB showed clear yield maxima at 1.5 g/l ammonium chloride (C:N ratio = 21.5) and 1.4 g/l ammonium sulfate (C:N ratio = 28.3). Note that all vertically aligned data in the upper and the lower parts of Fig. 4 represent the same C:N ratio, but with different concentrations of the two salts. The yields were relatively more sensitive to changes in the concentration of ammonium sulfate (Fig. 4), possibly because of differences in the bioavailability as a result of differences in the dissociation constants of the two salts. The specific product yield coefficient, $Y_{P/X}$, was relatively less sensitive to the C:N ratio (Fig. 4); the C:N values for best PHB yields did not give the highest value of $Y_{P/X}$. Maximum biomass and PHB concentrations were obtained with ammonium sulfate, hence it was used in all subsequent work. Sulfur may have been a growth-limiting factor when ammonium chloride was used.

Using an optimal C:N value of 28.3 (Fig. 4), two additional nitrogen sources—ammonium nitrate and urea—were tested. Other nutrients were as listed in Table 1 for Medium 1. The results for all nitrogen sources are summarized in Fig. 5. In comparison to ammonium sulfate (the best nitrogen source), the biomass yield in ammonium nitrate-supplemented medium was lower by a factor of 2.3, and the

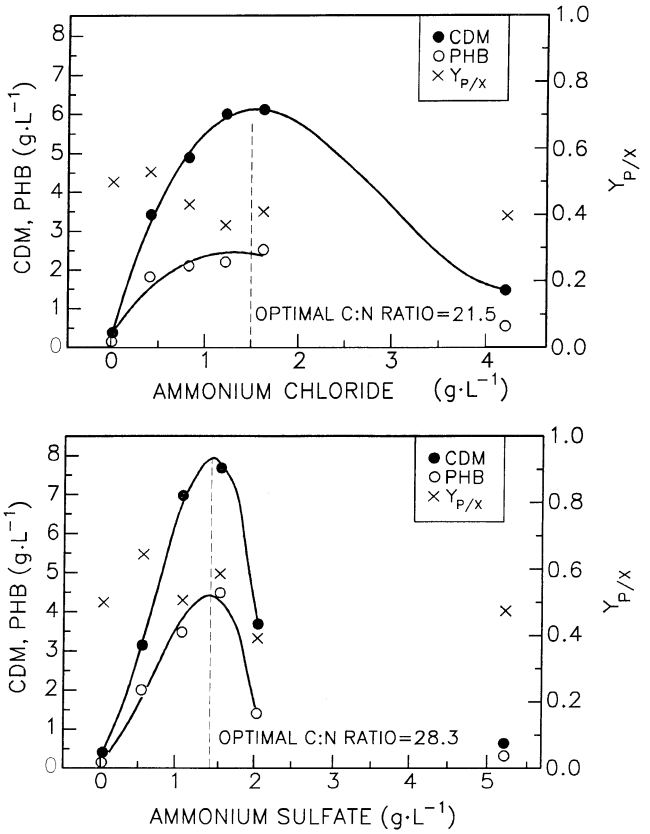

Fig. 4. Effects of the type of nitrogen source and the carbon-to-nitrogen ratio on biomass (CDM), PHB, and the product yield coefficient ($Y_{P/X}$) in Medium 1.

product yield was lower by a factor of 1.7. Apparently with ammonium nitrate, only the nitrogen of the ammonium was bioavailable, and enzymes for assimilation of nitrate were not synthesized. As shown in Fig. 5, *A. latus* could not metabolize urea as the sole nitrogen supply: the growth and PHB yield were comparable to the that when no nitrogen source was used.

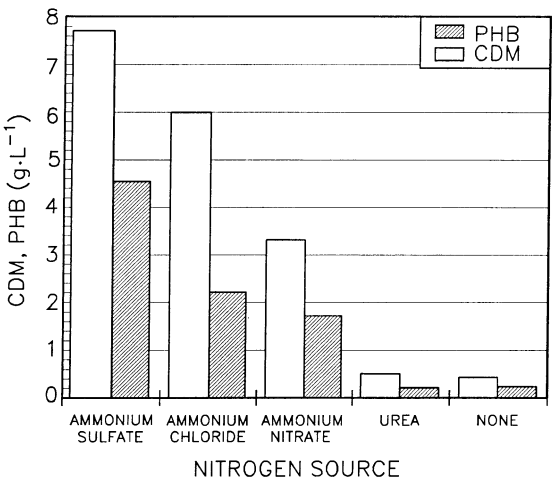

Fig. 5. Effect of the nitrogen source on biomass (CDM) and PHB yields at the optimal carbon-to-nitrogen ratio of 28.3.

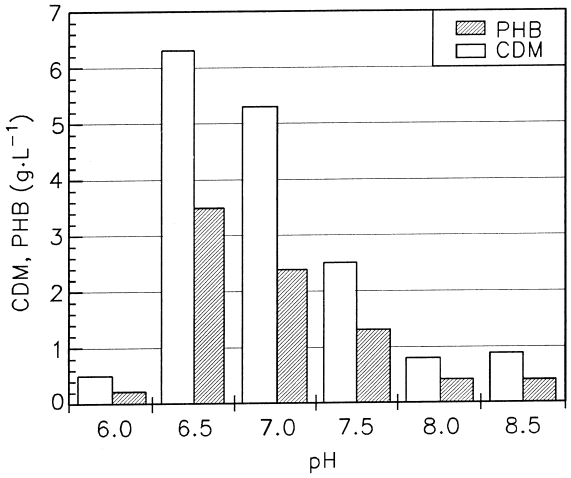

Fig. 6. Effect of initial culture pH on biomass (CDM) and PHB yields in Medium 1.

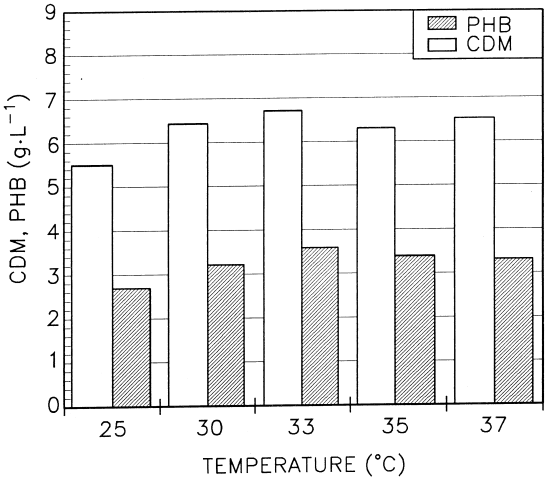

Fig. 7. Effect of culture temperature on the biomass (CDM) and PHB yields.

3.4. Influence of initial pH and temperature

The influence of initial culture pH on biomass yield and PHB production is shown in Fig. 6. The results shown were obtained in Medium 1 (Table 1) with the initial pH having been adjusted by dropwise addition of concentrated hydrochloric acid or sodium hydroxide as needed. Clearly, as shown in Fig. 6, an initial pH value of 6.5 gave the best results; pH values that differed even slightly from the optimum reduced the culture performance. These results are consistent with Palleroni and Palleroni [14],who recommended a pH range of 6.0–7.5 for microbial growth. The initial pH value may have affected the bioavailability of some of the trace elements. Note that acidic pHs as low as pH 5.5 developed during culture do not seem to affect *A. latus*; thus in Fig. 1, the exponential growth pattern is unaffected by the pH drop.

The effect of temperature on growth and PHB yield is shown in Fig. 7. From the data shown, 33°C appears to be the optimal temperature for growth and PHB synthesis; however, over the 25–37°C range, the temperature effect was negligible in view of the reproducibility of the measurements, which was ± 0.1 g/l.

3.5. Media development by factorial design

The factorial design approach to medium development relies on three stages of experimentation: screening, optimization, and verification. Screening experiments include many variables, but provide little information per variable. Screening aims at problem reduction, a determination as to which few process variable have the greatest impact on performance. Optimization experiments are designed to provide in-depth information about a few variables identified during screening as having the greatest impact on performance. Finally, verification experiments are used to validate the results under specific experimental conditions. The three levels of experimentation used in this study are detailed separately in the following sections.

3.5.1. Screening experiments

The screening experiments were designed to evaluate the impact of five factors, concentration of the carbon source, concentration of the nitrogen source, culture pH, IS, and the concentration of TES, at three levels as noted in Table 2. A two-level fractional factorial design was employed. Thus, for k factors, there were 2^{k-1} possible combinations of factors, or 16 experimental runs. In comparison to a full factorial design, which would have required 2^k experimental combinations, the two-level fractional factorial design FF0516 (i.e. 5 factors, 16 combinations) reduced the experimental effort at the expense of reduced ability to identify some higher order interactions among factors. The specific combinations of factors used in 16 runs and the resulting PHB yield (response) are noted in Table 3. The Table also lists three additional runs 17–19 that were included to check reproducibility. The runs were conducted in a randomized order to guard against systematic bias. The FF0516 design enabled the estimation of the principal effect (i.e. how the factor influenced the PHB yield) of each factor, as well as synergistic or antagonistic effects of two-factor interactions.

The PHB yields obtained in the various runs are also

Table 2
Factor levels for the screening experiments

Factor	Units	Low level	High level	Center point
C-source	g/l	15.0	25.0	20.0
N-source	g/l	1.5	3.0	2.25
pH	—	6.5	7.5	7.0
Ionic strength	g/l	4.0	12.0	8.0
TES	ml/l	1.0	3.0	2.0

Table 3
Factor values and response of screening runs

Run	Experimental factors					Response
	C-source (g/l)	N-source (g/l)	pH	Ionic strength (g/l)	TES (ml/l)	PHB (g/l)
1	15	1.5	6.5	4	3	2.48
2	15	1.5	6.5	12	1	0.73
3	15	1.5	7.5	4	1	2.19
4	15	1.5	7.5	12	3	1.04
5	15	3	6.5	4	1	1.75
6	15	3	6.5	12	3	1.14
7	15	3	7.5	4	3	2.50
8	15	3	7.5	12	1	1.24
9	25	1.5	6.5	4	1	2.19
10	25	1.5	6.5	12	3	1.17
11	25	1.5	7.5	4	3	2.41
12	25	1.5	7.5	12	1	0.93
13	25	3	6.5	4	3	2.45
14	25	3	6.5	12	1	0.98
15	25	3	7.5	4	1	2.04
16	25	3	7.5	12	3	1.41
17	20	2.25	7	8	2	1.58
18	20	2.25	7	8	2	1.49
19	20	2.25	7	8	2	1.53

noted in Table 3. In the runs noted, the values of the various factors were set close to the optimal settings derived from the preliminary shake flask experiments detailed previously. In all cases, the IS parameter was based on the concentration of the phosphate buffer components Na_2HPO_4 and KH_2PO_4. Both buffer components were used in equal amounts. An alternating pattern of high and low PHB yields is obvious in Table 3. This pattern corresponds to the high/low variation of IS (Table 3). Clearly, IS has a strong effect on PHB productivity. Also conspicuous in Table 3 are the three runs 17–19 with similar PHB yields. Those runs were included in the factorial design as additional center points. (A center point is an observation for which all factors have intermediate values between the high and low extremes.) Because each center point was run at the same conditions, an independent estimation of experimental error was possible, and replication of every run was unnecessary. Based on the standard deviation of center point, the maximum error in PHB yield was ± 0.07 g/l.

The interpretation of the results was based on estimates of how the various factors affected the response. The principal, or the main effect, of each factor was estimated as the difference in the average PHB yield in going from the low to the high setting of that factor, i.e. main effect = high level − low level. Two-factor interactions were estimated as the average of the runs in which both factors were at extreme values, minus the average of the runs in which the factor levels were mixed, i.e. interaction effect = average of high and low level − average of mixed level. The estimated main effects and the two-factor interactions are shown as a Pareto chart in Fig. 8. Clearly, for the ranges of variations tested, concentration of trace elements and the IS were the major influences on the PHB yield. Increasing TES had a beneficial effect on yield, whereas increasing IS was detrimental.

By using multiple regression analysis, the observed PHB yield was correlated with the experimental variables as follows: PBH yield = $1.645 - 0.586 \times \text{IS} + 0.159 \times \text{TES} + 0.054 \times \text{pH} + 0.032 \times C + 0.023 \times N$. For this equation, the regression coefficient (R) value was 0.971, the R^2 was 0.944, the standard deviation (SD) was 0.160, the SD of the intercept was 0.038, and that of the coefficient was 0.041. The R^2 value confirmed that the variability in the PHB yield could be associated to the experimental factors to

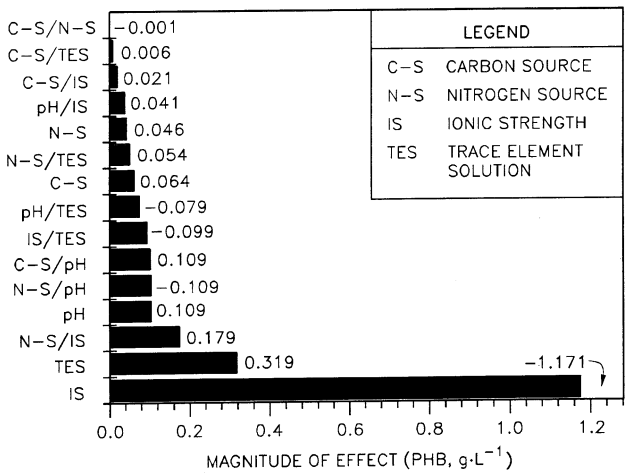

Fig. 8. Pareto chart for the screening experiments. The effects of individual factors and two-factor combinations are noted for the carbon source concentration, the nitrogen source concentration, the amount of TES, the IS, and the pH.

Table 4
Factor levels for the optimization experiments

Factor	Levels				
	$-\alpha$	−1	0	+1	$+\alpha$
Ionic strength (g/l)	0.59	1	2	3	3.41
TES (ml/l)	2.59	3	4	5	5.41

the extent of 94.4%. The coefficients in the equation can also be estimated from the Pareto chart (Fig. 8) by dividing the corresponding value of an effect by 2. The *C* and *N* in the equation are the concentrations (g/l) of the carbon- and the nitrogen-sources, respectively.

In view of the results of the screening experiments, higher levels of TES and lower values of IS were indicated for the future optimization runs. The three other factors—pH, the carbon source, and the nitrogen source—did not have a major impact on yield; hence, for future optimization, the values of those factors could be set to the low economic levels.

3.5.2. Optimization and verification

Two factors—IS (buffer concentration) and TES (concentration of the trace element solution)—survived the initial screening for significant variables. The scope of the problem thus reduced and an optimization experiment became feasible. An orthogonal central composite design CC0211 for two independent factors, each at five levels with four α-points and three replicates at the center, was used for optimizing PHB production in shake flasks. The coding used for the two factors in the optimization runs is noted in Table 4. The position of the α-points was established statistically to ensure optimal coverage of the experimental space. The layout of points in the experimental space is depicted in Fig. 9. The concentrations of sucrose and ammonium sulfate were fixed at 20 g/l and 1.5 g/l, respectively, and the initial pH was 7.0. The levels of the two variable factors and the resulting PHB yields for the 11 experimental runs are noted in Table 5. At 2.97 g/l, the average PHB yield in the optimization runs was 1.8-fold greater than the average yield in the screening experiment; hence, confirming that the three fixed parameters, i.e. pH, C-source level, and N-source concentration, were at suitable values. Based on the standard deviation of the center points (runs 9–11 in Table 5) the maximum error in the PHB yields was ± 0.06 g/l.

The results of the optimization runs are depicted in Fig. 10. The highest PHB yield occurred at 1 ml/l TES and 4 g/l phosphate buffer concentration, thus confirming the strong negative effect of IS, but not the positive effect of TES

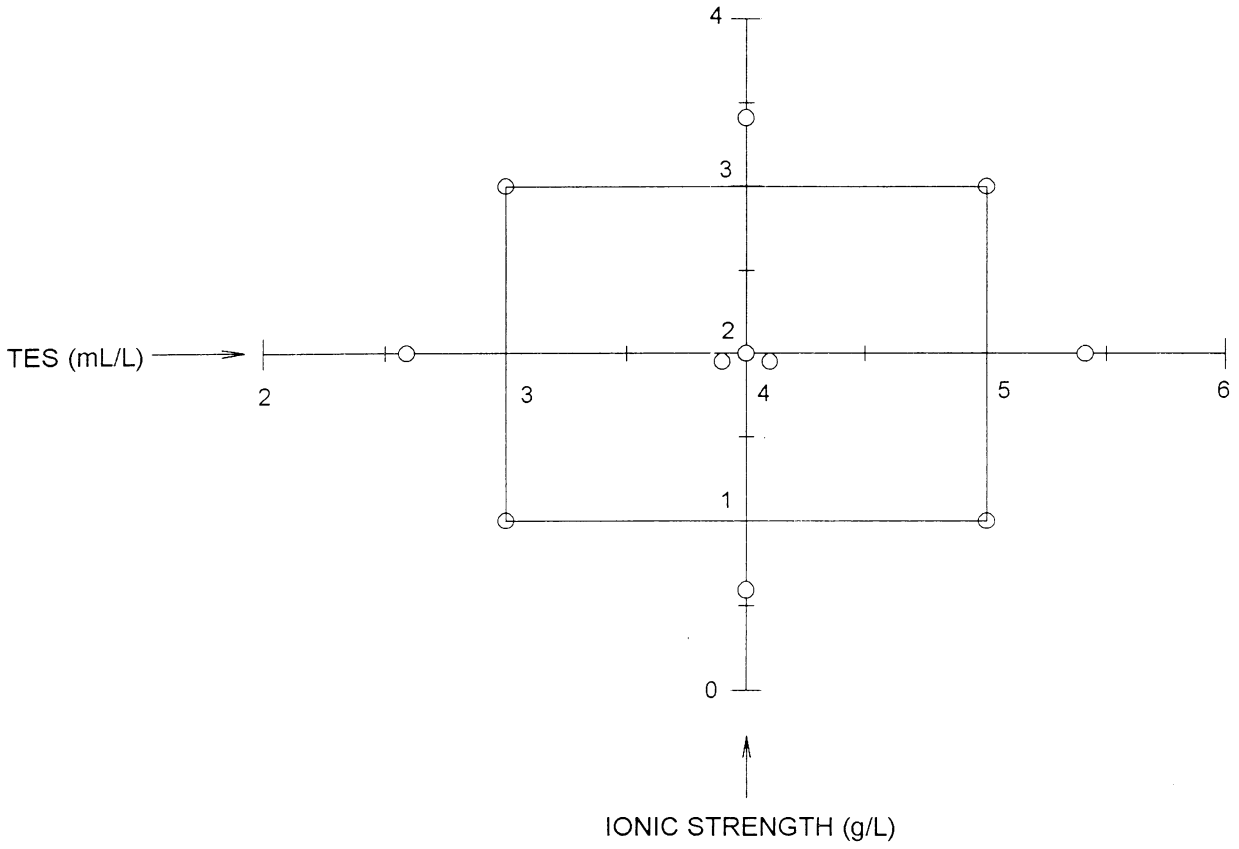

Fig. 9. Layout of points in the experimental space.

Table 5
Factor levels and the response of the optimization runs

Run[a]	Ionic strength	TES	PHB (g/l)
1	−1	−1	3.49
2	−1	+1	2.22
3	+1	−1	3.45
4	+1	+1	2.47
5	−1.41	0	2.87
6	+1.41	0	2.40
7	0	−1.41	3.54
8	0	+1.41	2.73
9	0	0	3.23
10	0	0	3.14
11	0	0	3.13

[a] Runs were conducted in a randomized order to guard against systematic bias.

observed in the prior screening experiments. However, the magnitude of the TES effect was small in comparison to that of the IS (see coefficients in the first equation).

The relationship among the response PHB yield and the two investigated factors could be represented as a response surface plot as shown in Fig. 11. The response surface was generated by using the quadratic polynomial $z = A + B \times x + C \times y + D \times x^2 + E \times y^2 + F \times x \times y$. In this equation, z is the predicted response, A is the intercept, the x and y terms stand for the two experimental factors, and the squared terms represent the curvature of the surface. The two-factor interaction is represented by the $x \times y$ term. The polynomial regression of the experimental data yielded the following response surface model: PBH yield $= 3.167 - 0.057 \times \text{IS} - 0.425 \times \text{TES} - 0.261 \times \text{IS}^2 - 0.010 \times \text{TES}^2 + 0.073 \times \text{IS} \times \text{TES}$.

The various goodness-of-fit indicators for this equation were $R = 0.998$, $R^2 = 0.997$, SD = 0.104, SD of the intercept = 0.088, and the parameter SD = 0.095. Based on the R^2 value, the variation in the PHB yield could be described by the last equation with a certainty of 99.7%.

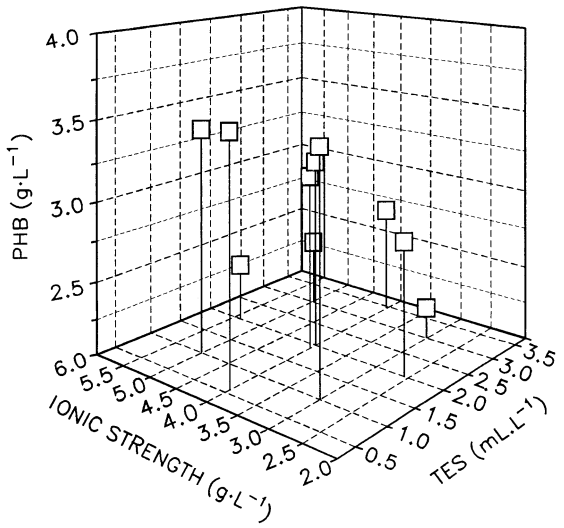

Fig. 10. The PHB yield obtained in optimization experiments for various combinations of IS and TES.

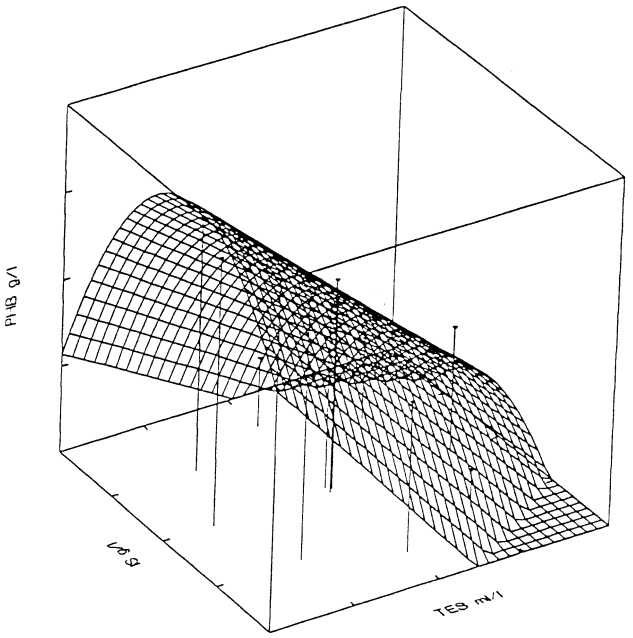

Fig. 11. The response surface plot of PHB yield as a function of IS and TES.

4. Conclusions

Batch cultures of *A. latus* ATCC 29713 were investigated and optimized for the production of the microbial thermoplastic PHB. The typical culture characteristics were: 0.075/h maximum specific growth rate, 0.38 g/l h maximum specific sucrose consumption rate, and 0.15 g/l h maximum specific PHB production rate. The following specific conclusions were reached:

1. The PHB production is growth associated. Degradation of the formed PHB commences with the onset of the stationary phase; hence, timing the harvest is essential to prevent loss of the produced PHB.
2. The type of nitrogen source and the carbon-to-nitrogen ratio affect biomass and PHB yields. Ammonium sulfate is a suitable nitrogen source; ammonium nitrate is unsatisfactory; and urea is not metabolized to a significant extent. When using ammonium nitrate and sucrose, the optimal C:N ratio is 28.3. The sensitivity of the biomass and PHB yields to changes in C:N depends on the type of the nitrogen source used.
3. A suitable complement of trace elements is essential to attaining high PHB productivity and yield; however, excessively high or excessively low concentrations of trace elements reduce productivity.

4. The initial pH of the culture medium significantly affects biomass and PHB productivities, presumably by affecting the bioavailability of one or more nutrients. The optimal initial pH is 6.5.
5. The optimal temperature for growth and PHB formation appears to be 33°C, although over the 25–37°C range, the effect of temperature is negligibly small.
6. The IS of the medium has a strong effect on PHB production. A suitable IS value is 4 g/l.
7. Through a statistically designed optimization, the PHB yield could be increased from an average of 1.64 g/l in screening experiments to an average of 2.97 g/l in the optimization experiments; i.e. a 1.8-fold yield enhancement.

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