Research Article

Bioprocess Engineering Aspects of Biopolymer Production by the Cyanobacterium *Spirulina* Strain LEB 18

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Microbial biopolymers can replace environmentally damaging plastics derived from petrochemicals. We investigated biopolymer synthesis by the cyanobacterium *Spirulina* strain LEB 18. Autotrophic culture used unmodified Zarrouk medium or modified Zarrouk medium in which the NaNO_3 content was reduced to 0.25 g L^{-1} and the NaHCO_3 content reduced to 8.4 g L^{-1} or increased to 25.2 g L^{-1} . Heterotrophic culture used modified Zarrouk medium containing 0.25 g L^{-1} NaNO_3 with the NaHCO_3 replaced by 0.2 g L^{-1} , 0.4 g L^{-1} , or 0.6 g L^{-1} of glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) or sodium acetate (CH_3COONa). Mixotrophic culture used modified Zarrouk medium containing 0.25 g L^{-1} NaNO_3 plus 16.8 g L^{-1} NaHCO_3 with the addition of 0.2 g L^{-1} , 0.4 g L^{-1} , or 0.6 g L^{-1} of glucose or sodium acetate. The highest biopolymer yield was 44% when LEB 18 was growing autotrophically in media containing 0.25 g L^{-1} NaNO_3 and 8.4 g L^{-1} NaHCO_3 .

1. Introduction

Instability in the international price of oil and natural gas, coupled with geopolitical factors and environmental concerns, has led to the need to substitute nonrenewable petroleum products with those based on renewable resources. The production of polymers from petrochemicals is the second major use of oil after their utilization as a source of energy [1]. Biopolymers can be produced using biofixation of carbon dioxide by cyanobacterium and could reduce both dependency on petroleum and carbon dioxide emissions [2].

Petrochemical-based plastics are resistant to degradation and most eventually end up in sanitary landfills, where they often compromise the circulation of gas and liquids and thus the decomposition of other materials within the site and may even make it unstable. Since landfill sites are becoming scarce, one solution is to substitute recalcitrant petrochemical-based plastics with biopolymers that do not cause such problems [3].

Polyhydroxyalkanoates (PHAs), including polyhydroxybutyrate (PHB), polyhydroxypropionate (PHP), and polyhydroxyvalerate (PHV), are bacterial or cyanophyte aliphatic polyesters which have thermoplastic, mechanical, and physical properties similar to polypropylene. PHAs are biocompatible, recyclable, and biodegradable and produce zero toxic waste since they biodegrade into carbon dioxide and water by microbial attack in about three months to one year [4, 5]. These polymers have a high degree of polymerization and are crystalline, optically active, isotactic, and insoluble in water [6, 7].

Cyanobacteria have the potential for the production of biopolymers and their yield can be increased by stressing the culture via nutrient limitation or other means, use of recombinant strains [8], control of metabolic flux, and the use of different bioreactor types [1]. Some cyanophytes can adapt their metabolism during nutrient limitation [9], with biopolymer synthesis generally occurring when the carbon and energy source is present at normal levels or in

excess while at least one other nutrient is limiting, nitrogen, phosphorus, magnesium, and iron being among the most common limiting nutrients [3]. Unlike crop plants, the cultivation of cyanobacteria does not require the use of large areas of ground and can occupy areas inappropriate for agriculture and thus does not compete with food production [10].

We investigated methods to stimulate the synthesis of biopolymer by the cyanophyte *Spirulina* strain LEB 18 cultivated with different carbon sources and reduced nitrogen levels.

2. Material and Methods

2.1. Microorganism and Culture Media. The cyanobacterium *Spirulina* strain LEB 18 [11] was maintained in unmodified Zarrouk liquid mineral salts medium, containing mineral salts (K_2HPO_4 , K_2SO_4 , NaCl, $MgSO_4$, $CaCl_2$, $FeSO_4$, and EDTA) and 16.8 g L^{-1} of sodium bicarbonate ($NaHCO_3$) as the carbon source and 2.5 g L^{-1} of sodium nitrate ($NaNO_3$) as the nitrogen source [12].

Autotrophic culture used unmodified Zarrouk medium or modified Zarrouk medium in which the $NaNO_3$ content was reduced to 0.25 g L^{-1} and the $NaHCO_3$ content was reduced to 8.4 g L^{-1} or increased to 25.2 g L^{-1} . Heterotrophic culture used modified Zarrouk medium containing 0.25 g L^{-1} $NaNO_3$ with the $NaHCO_3$ replaced by 0.2 g L^{-1} , 0.4 g L^{-1} , or 0.6 g L^{-1} of glucose ($C_6H_{12}O_6$) or sodium acetate (CH_3COONa). Mixotrophic culture used modified Zarrouk medium containing 0.25 g L^{-1} $NaNO_3$ plus 16.8 g L^{-1} $NaHCO_3$ with the addition of 0.2 g L^{-1} , 0.4 g L^{-1} , or 0.6 g L^{-1} of glucose or sodium acetate. The growth media components are summarized in Table 1.

2.2. Experimental Conditions. Cultures were grown in 2 L closed photobioreactors with a working volume of 1.5 L and continuous agitation provided by the injection of sterile air. The inocula were adapted for 20 days in their respective culture media. The initial biomass concentration was 0.15 g L^{-1} and the initial volume was 1.5 L. The cultures were maintained at 30°C for 15 days under a 12 h photoperiod and 3200 lux provided by 40 W daylight-type fluorescent lamps.

2.3. Analytical Assays. Samples (10 mL) were collected aseptically at the same time every 24 h and the pH was measured at the same time using a Q400H digital pH meter (Quimis, Brazil) according to the methodology of the Association of Analytical Communities [13]. Growth of LEB 18 was estimated by measuring the optical density at 670 nm in a Q798DRM spectrophotometer (Quimis, Brazil) and comparing the reading to a calibration curve relating optical density to biomass [14].

2.4. Determination of Kinetic Parameters. Growth curves of LEB 18 biomass against time were plotted and the following parameters calculated: maximum biomass concentration ($X_{\max}$, g L^{-1}); productivity (P , $\text{g L}^{-1}\text{ d}^{-1}$), calculated as $P = X_t - X_0 / t - t_0$ [15], in which X is the biomass (g L^{-1}) at time

t (d) and X_0 is the biomass (g L^{-1}) at time t_0 (d), maximum productivity ($P_{\max}$, $\text{g L}^{-1}\text{ d}^{-1}$) being the productivity at $X_{\max}$, and specific growth rate (μ , d^{-1}) was calculated as $\mu_{\max} = 0.693/t_d$, where 0.693 is the natural logarithmic of 2 and t_d is the biomass doubling time [16], the maximum specific growth rate being $\mu_{\max}$ (d^{-1}).

2.5. Extraction Biopolymers. After fifteen days of cultivation, biopolymers were extracted from each photobioreactor run by centrifuging the culture media to precipitate the LEB 18 biomass, to which was added 10% to 12% v/v sodium hypochloride solution and the mixture recentrifuged. The supernatant was discarded and the precipitate was washed with distilled water, then recentrifuging and again discarding the supernatant and adding acetone to precipitate the biopolymer. Then, it was dried in an oven at 35°C for 48 h.

The yield (η) was calculated as grams of biopolymer (bp) per gram of biomass (bm) from the equation $\eta = m_{bp} \times 100/m_{bm}$, where m_{bp} is the final amount of biopolymers in grams extracted from LEB 18 and m_{bm} is the amount of LEB 18 biomass in grams from which the biopolymers were extracted.

2.6. Statistics Analysis. Where appropriate, the data was subjected to analysis of variance (ANOVA) and the Tukey test at $P = 95\%$. All reagents were of at least analytical grade. Where appropriate, percentages are weight dry for weight dry (w/w) unless otherwise indicated.

3. Results and Discussion

The LEB 18 data is given in Table 1 and the growth curves are given in Figures 1 and 2, from which it can be seen that the different culture media were broadly similar regarding the various carbon sources (Table 1). The curves show no lag phase (Figures 1 and 2) because the inocula were preadapted for each culture medium, thus allowing LEB 18 to enter directly into the exponential growth phase. Growth, measured as $X_{\max}$, was not limited during the exponential phase, even in the experiments containing the maximum concentration of carbon (16.8 g L^{-1} of glucose or sodium acetate, 25.2 g L^{-1} of $NaHCO_3$).

The culture which obtained the highest $X_{\max}$ value (0.86 g L^{-1}) used $NaHCO_3$ (8.4 g L^{-1} and 16.8 g L^{-1}) as the carbon source and 0.25 g L^{-1} $NaNO_3$ as the nitrogen source (Table 1, Figure 2). The highest $\mu_{\max}$ value (0.12 d^{-1}) occurred with unmodified Zarrouk medium (Table 1). However, there was no statistical difference between any of the culture media regarding $X_{\max}$ values (Table 1). Since bicarbonate is the carbon source in unmodified Zarrouk medium, LEB 18 already expressed the enzymes necessary for carbon assimilation from this carbon source, enabling more growth than glucose or acetate.

The experiments were concluded after 15 days when the cultures entered the stationary phase. In general, the production of intracellular biocompounds generally occurs

TABLE 1: Metabolic type, carbon sources, and growth parameters for *Spirulina* strain LEB 18 cultivated with different carbon sources. The same superscript letter in the same column indicates no significant difference at the 95% confidence level.

Metabolic type and carbon sources		Carbon source concentration (g L ⁻¹)	Growth parameters			Biopolymers yield (η, grams of biopolymer per gram of LEB 18 biomass)
		Maximum cell concentration (X _{max} , g L ⁻¹)	Maximum specific growth rate (μ _{max} , d ⁻¹)	Maximum cell productivity (P _{max} , g L ⁻¹ d ⁻¹)		
Glucose (see column 2 for concentration), 16.8 g L ⁻¹ NaHCO ₃ , and 0.25 g L ⁻¹ NaNO ₃	0.2	0.74 ± 0.12 ^a	0.06 ± <0.006 ^{abc}	0.10 ± 0.01 ^{bc}	11.33 ± 1.09 ^a	
	0.4	0.72 ± 0.11 ^a	0.06 ± <0.006 ^{bcd}	0.13 ± 0.02 ^{ab}	12.30 ± 0.22 ^a	
	0.6	0.69 ± 0.16 ^a	0.04 ± <0.006 ^a	0.17 ± 0.01 ^a	10.05 ± 1.34 ^a	
Sodium acetate (see column 2 for concentration), 16.8 g L ⁻¹ NaHCO ₃ , and 0.25 g L ⁻¹ NaNO ₃	0.2	0.65 ± 0.15 ^a	0.09 ± <0.006 ^{ef}	0.05 ± 0.02 ^{de}	11.58 ± 0.27 ^a	
	0.4	0.69 ± 0.10 ^a	0.09 ± <0.006 ^{def}	0.08 ± 0.02 ^{cde}	11.32 ± 3.17 ^a	
	0.6	0.77 ± 0.17 ^a	0.08 ± <0.006 ^{cdef}	0.09 ± 0.00 ^{bcd}	11.93 ± 0.69 ^a	
NaHCO ₃ (see column 2 for concentration) and 0.25 g L ⁻¹ NaNO ₃	8.4	0.86 ± 0.01 ^a	0.07 ± 0.01 ^{cdef}	0.05 ± 0.01 ^{cde}	44.19 ± 0.15 ^b	
	16.8	0.86 ± 0.17 ^a	0.06 ± 0.01 ^{bcd}	0.06 ± 0.02 ^{cde}	40.00 ± 1.88 ^b	
	25.2	0.79 ± 0.01 ^a	0.09 ± <0.006 ^{def}	0.05 ± 0.00 ^{de}	37.57 ± 3.16 ^b	
Glucose (see column 2 for concentration) and 0.25 g L ⁻¹ NaNO ₃	0.2	0.73 ± 0.06 ^a	0.08 ± <0.006 ^{def}	0.08 ± 0.00 ^{cde}	11.31 ± 0.20 ^a	
	0.4	0.64 ± 0.07 ^a	0.05 ± <0.006 ^{ab}	0.10 ± 0.01 ^{bcd}	10.38 ± 2.88 ^a	
	0.6	0.65 ± 0.01 ^a	0.07 ± <0.006 ^{cde}	0.13 ± 0.02 ^{ab}	10.77 ± 0.47 ^a	
Sodium acetate (see column 2 for concentration) and 0.25 g L ⁻¹ NaNO ₃	0.2	0.65 ± 0.11 ^a	0.09 ± 0.01 ^f	0.04 ± <0.006 ^e	7.64 ± 0.46 ^a	
	0.4	0.63 ± 0.05 ^a	0.07 ± 0.01 ^{cdef}	0.05 ± 0.01 ^{cde}	8.18 ± 0.70 ^a	
	0.6	0.68 ± 0.03 ^a	0.08 ± <0.006 ^{cdef}	0.04 ± <0.006 ^{de}	7.83 ± 0.01 ^a	
Unmodified Zarrouk media containing 16.8 g L ⁻¹ NaHCO ₃ and 2.5 g L ⁻¹ NaNO ₃ (control)		16.8		0.12 ± <0.006 ^g	0.04 ± <0.006 ^{de}	10.26 ± 0.60 ^a

FIGURE 1: Growth curves for LEB 18 in modified Zarrouk media containing a reduced level of nitrogen ($0.25 \text{ g L}^{-1} \text{ NaNO}_3$) and the following carbon sources: (a) $16.8 \text{ g L}^{-1} \text{ NaHCO}_3$ plus glucose, (b) $16.8 \text{ g L}^{-1} \text{ NaHCO}_3$ plus sodium acetate, (c) glucose, and (d) sodium acetate. The glucose and acetate concentrations used were 0.2 g L^{-1} (+), 0.4 g L^{-1} (■), and 0.6 g L^{-1} (●).

FIGURE 2: Growth curves for LEB 18 in modified Zarrouk media containing a reduced level of nitrogen ($0.25 \text{ g L}^{-1} \text{ NaNO}_3$) and NaHCO_3 at the following concentrations: 8.4 g L^{-1} (+), 16.8 g L^{-1} (■), and 25.2 g L^{-1} (●). Results are also shown for unmodified Zarrouk medium containing $0.25 \text{ g L}^{-1} \text{ NaNO}_3$ and $16.8 \text{ g L}^{-1} \text{ NaHCO}_3$ (▲).

in the stationary phase when microbial growth ceases. However, biopolymers such as PHAs serve as intercellular energy reserves, because of which their production generally occurs during the exponential phase in parallel with whatever factor is used to measure cell growth, which in our case was the increase in LEB biomass. This aids in the survival of the producing microorganism, which can use PHAs for survival in the later stages of growth when nutrients become limiting.

We carried out preliminary tests in which we extracted biopolymers every 5 days for 30 days (data not shown) and observed that the end of the exponential phase and the beginning of the stationary phase occurred at around 15 days after inoculation and that biopolymer yields were highest at this time. Studies by other workers of biopolymer production by cyanophytes have shown higher yields during the stationary phase, with the cyanophyte *Nostoc muscorum* producing 8.6% [17] and the cyanophyte *Synechocystis* sp. strain PCC 6803 producing 45% during this phase.

The quantity of nitrogen available is known to directly influence biopolymer synthesis and in our experiments the biopolymer yield increased fourfold from 10.26% to 40% when the sodium nitrate content was reduced from 2.5 g L^{-1} to 0.25 g L^{-1} , 10% of its original value (Table 1).

Unmodified Zarrouk medium contained $16.8 \text{ g L}^{-1} \text{ NaHCO}_3$ plus $2.5 \text{ g L}^{-1} \text{ NaNO}_3$ and produced a yield (η) of 10.26%, probably due to the high level of nitrogen (Table 1). However, in modified Zarrouk media containing the same quantity of NaHCO_3 but only $0.25 \text{ g L}^{-1} \text{ NaNO}_3$ the biopolymer yield was 40%. Furthermore, when growing in modified Zarrouk medium with reduced nitrogen, $\mu_{\max} = 0.06 \text{ d}^{-1}$ was half the rate occurring with unmodified Zarrouk medium, where $\mu_{\max} = 0.12 \text{ d}^{-1}$ (Table 1). It is known that $\mu_{\max}$ values are higher for actively growing cells, which was the case in unmodified Zarrouk medium,

and that when energy is diverted for the synthesis of storage compounds, including biopolymers, lower values of $\mu_{\max}$ occur. It is interesting to note that while there was no statistically significant difference ($P > 0.05$) between the $\mu_{\max}$ values for the three versions of modified Zarrouk medium, there was a statistically significant difference ($P < 0.05$) between these and the unmodified Zarrouk medium (Table 1).

It has been reported that the eukaryotic algae *Nannochloropsis* growing in wastewater supplemented with F/2 medium showed $\mu_{\max} = 0.33 \text{ d}^{-1}$ and a lipids content of 30% but when $\mu_{\max} = 0.54 \text{ d}^{-1}$ the lipid content was only 23% [18]. Other workers have reported $\mu_{\max} = 0.13 \text{ d}^{-1}$ during semicontinuous cultivation of the cyanophyte *Cyanobium* in BG-11 medium with the addition of 0.4 g L^{-1} of NaHCO_3 [19].

The maximum productivity ($P_{\max}$, Table 1) occurred between the second and the eighth days of culture, probably because at this stage nutrients were available in greater concentrations and LEB 18 had been preadapted to the culture media (Figure 1(a)). Furthermore, during this initial period, little PHB would have been produced and the cultures energy resources could have been directed mainly to biomass production. In the trials with inorganic carbon, $P_{\max}$ occurred between the fifth and the eighth days of culture. Our $P_{\max}$ values ranged from $0.04 \text{ g L}^{-1} \text{ d}^{-1}$ to $0.17 \text{ g L}^{-1} \text{ d}^{-1}$ (Table 1), similar to the $P_{\max} = 0.07 \text{ g L}^{-1} \text{ d}^{-1}$ reported for *Cyanobium* growing in BG-11 media [19] and $P_{\max} = 0.08 \text{ g L}^{-1} \text{ d}^{-1}$ for LEB 18 growing in medium containing $10 \text{ g L}^{-1} \text{ NaHCO}_3$ [2].

The medium which presented the highest biopolymer yield (η) contained $8.4 \text{ g L}^{-1} \text{ NaHCO}_3$ and 0.25 g L^{-1} of nitrogen, and there appeared to be a reduction in yield as the NaHCO_3 concentration increased in the reduced nitrogen media, although the differences were not statistically significant (Table 1). However, it is known that although high levels of carbon are needed to stimulate the synthesis of biopolymers the excess can also inhibit production because although acetyl-CoA can enter the PHB biosynthesis pathway free acetyl-CoA inhibits the enzyme β -ketothiolase which is responsible for PHB synthesis.

The addition of the organic carbon sources, glucose, and sodium acetate did not stimulate either biomass production or biopolymer synthesis. Furthermore, there was no significant difference ($P < 0.05$) in biopolymer yield when adding sodium bicarbonate to cultures containing glucose or sodium acetate. It is known that when two endogenous carbon sources are available a microorganism usually gives preference to one of them. Since LEB 8 is generally maintained in unmodified Zarrouk medium containing sodium bicarbonate it already has the necessary pathways to utilize this nutrient, making two sources of carbon superfluous. It has been reported that *Spirulina platensis* UMACC 161 can produce 10% PHB when using a medium containing 9 g L^{-1} of NaHCO_3 plus 0.5% sodium acetate without the presence of nitrogen but when the media contained NaHCO_3 plus CO_2 the PHB yield was around 3% [20]. Yields of 47% PHB have been reported for the cyanophyte *Nostoc muscorum* growing

in BG-11 medium containing glucose and sodium acetate but without a nitrogen source [21].

The biopolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) has been produced from *Nostoc muscorum* using various carbon sources (acetate, fructose, glucose, propionate, and valerate) with the PHBV yield being 28% when using 0.4% w/v acetate and 26% when using 0.4% w/v glucose, the yield rising to 60% when the carbon source was 0.4% acetate or valerate and the nitrogen source was restricted [22]. Lower yields of 9.5% have been reported for the cyanophyte *Synechocystis* sp. strain PCC 6803 growing in BG-11 medium. However, when cultivated with sodium acetate, the yield increased to 14.6% under nitrogen limitation and 25.7% under phosphate limitation [23].

The yield of microbial biopolymers is quite variable and depends on the composition of the medium used. When we grew LEB 18 on sodium bicarbonate, glucose, or sodium acetate along with different concentrations of nitrogen we obtained biopolymer yields varying from 7.64% to 44.19% (Table 1). The cyanophyte *Aulosira fertilissima* has been investigated for PHB production using media containing 0.2% w/v to 0.4% w/v acetate or citrate plus from zero to $20 \text{ mg L}^{-1} \text{ K}_2\text{HPO}_4$ with an incubation period from 2 days to 14 days, with PHB yields ranging from 17% to 85.5% [24].

Bacteria produce large amounts of biopolymers in a short time; however, cyanobacteria have the advantage of using smaller amounts of nutrients due to photosynthesis, which uses the solar energy and transforms the carbon dioxide into oxygen, which is essential for humans.

Decreasing the quantity of nitrogen in the culture medium resulted in an increase in polymer production by LEB 18. Autotrophic growth in modified Zarrouk medium containing 16.8 g L^{-1} of NaHCO_3 and 0.25 g L^{-1} of NaNO_3 resulted in about 74% more biopolymer production than in unmodified Zarrouk medium (Table 1). It has been pointed out that cyanophyte *Spirulina* is a rich source of proteins [25], implying a large nitrogen requirement for growth. Under nitrogen limitation it is known that cyanophytes can divert carbon into other metabolic routes and produce biopolymers [21] to serve as carbon and energy storage compounds which can be reused when conditions become more favorable. The nitrogen content of the environment increases and the organism can produce proteins for cell growth rather than the storage lipids from which PHB derives.

4. Conclusions

In our experiments we found that the maximum biopolymer yield was 44.19% when LEB 18 was cultivated in modified Zarrouk medium containing 8.4 g L^{-1} of sodium bicarbonate and 0.25 g L^{-1} of sodium nitrate. The biopolymers produced by cyanobacteria have many uses because they present biocompatibility with mammalian cells and tissues and biodegradability. The main applications for biopolymers are in the food and medical areas, but they can also help reduce the environmental pollution generated by petrochemical-derived plastics.

This study shows that the cyanobacteria are potential sources of biopolymers, because they can use smaller amounts of nutrients and help to reduce the environmental pollution.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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