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Poly-β-hydroxybutyrate Production by Fast-Growing Rhizobia Cultivated in Sludge and in Industrial Wastewater

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Abstract In our study, the potential of producing polyhydroxybutyrate (PHB) by cultivating fast-growing rhizobia (*Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *phaseoli* and *R. leguminosarum* bv. *trifolii*) in sludge and in industrial wastewater was evaluated. Results confirmed the possibility of using sludge as media for rhizobial growth. During growth, substantial quantity of PHB was accumulated and yields varied depending on the media and rhizobial species. Growing in sludge, PHB production did not exceed 3.7% *w*/*w* for all strains at the end of experiment (after 72 h). During the growth of *S. meliloti*, PHB yield varied and the maximum value reached 7.27% *w*/*w* after 60 h, with 1% Total Suspend Solid (TSS) sludge. Alkaline sludge pre-treatment affects rhizobial growth but did not improve the PHB accumulation. While growing *S. meliloti* in industrial wastewater, the PHB yields varied and the highest value was obtained with slaughterhouse wastewater (10.7% w/w) after 35 h of growth. Therefore, this work shows the potential of exploiting PHB production by rhizobia growing in wastewater or sludge which could be applied to bioplastic industry, and confirms the potential of these recyclable wastes for high production of rhizobial cells useable for legumes inoculants production. This study provides an environmentally sound way of sludge and wastewater management and use in diverse biotechnological applications.

Keywords Poly-β-hydroxybutyrate · Fast-growing rhizobia · Sludge · Industrial wastewater

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Introduction

Biodegradable plastics represent a solution to environmental problems generated by the utilization of plastics from petrochemical sources, which have many undesirable proprieties such as durability and resistance to biodegradation. PHB is the most known polyhydroxyalkanoate (PHA). PHB is accumulated by a large number of bacteria under conditions of nutrient limitation and in the presence of an abundant source of carbon [1]. The accumulation of PHA at higher concentration has been observed in a variety of micro-organisms such as *Clostridium, Syntrophomonas, Pseudomonas,* and *Alcaligenes* genera. Some cyanobacteria produce PHB as well but at lower levels [2]. Some studies showed that rhizobia strains are able to accumulate PHB at a significant level [3, 4].

Generally, the nature and the proportion of polymer produced by bacteria are controlled by the carbon source used during culture [5]. Moreover, bioplastic utilization depends on the production costs and on polymer proprieties. Hence, 40 to 50% of the total production cost is related to the raw material [6]. In order to reduce the production cost, the use of cheaper carbon source is needed. In this perspective, many waste materials such as food wastes, potato starch wastewater, alpechin, and wastewater sludge [7, 8] have been used as substrates for bioplastic production. More recently, sludge generated by industrial and municipal wastewater-treatment processes, a worldly recyclable waste, has shown good potential to be used as a growth medium and as a carrier (dehydrated sludge) for rhizobia-based inoculant production (reviewed by [9]). Because rhizobia can accumulate PHB during growth, the purpose of this study was to evaluate the potential of producing PHB by fast-growing rhizobia cultivated in sludge and in industrial wastewater.

Materials and Methods

Sludge and Wastewater Sampling

Two types of industrial wastewater (starch and slaughterhouse wastewater from a plant located around Quebec region) and a secondary sludge (Quebec municipal wastewater-treatment plant) were sampled and stored at 4°C until their use. The pH was measured with a pH meter (Orion model 420A). Total suspended solid (TSS), total Kjeldahl nitrogen (TKN), phosphorus (P_t), and total organic carbon (TOC) were determined according to the Standard Methods [10].

Sludge Pre-treatment

Alkaline treatment was used to hydrolyze organic matter in sludge. The alkaline pre-treatment consists of 24 h hydrolysis of 100 ml sludge by increasing the pH to 12.0 with sodium hydroxide (2 N solution). The sludge TSS concentrations were 1% and 1.5% (obtained by concentration of the original sample). After treatment, the pH was adjusted to 7.0 with sulfuric acid (2 N solution).

Micro-organisms

Four rhizobia strains (Agriculture and Agri-Food Canada, Quebec, QC) were used in the present study: *Sinorhizobium meliloti* (A_2); *Rhizobium leguminosarum* bv *viciae* (USDA23370); *Rhizobium leguminosarum* bv *phaseolus* (USDA2671), and *Rhizobium*

leguminosarum bv *trifolii* (ATCC14480). Strains were maintained on yeast mannitol agar (YMA) slants at 4°C.

Inoculum Preparation

The inocula for the experiments were prepared by growing strains in 250-ml Erlenmeyer flasks containing 25 ml of the sterilized standard medium (YMB: yeast mannitol broth). The flasks were incubated at 30°C for 48 h on a rotary shaker at 200 rpm. The standard medium contained the following constituents (in grams per liter): K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; yeast extract, 1; and mannitol, 10.

Growth Experiments

Growth experiments were carried out in 500-mL Erlenmeyer flasks each containing 100 ml of standard medium. In order to stimulate the accumulation of PHB 1 ml l^{-1} of 0.9% (*w*/*v*) solution of ferric chloride was added to the standard medium [3]. Flasks were inoculated with 3% (*v*/*v*) inoculum. The conditions used in the experiments were the same as those used to prepare the corresponding inoculum. Samples, 0.5 to 2 ml, were drawn during growth for analyses. *S. meliloti* (A_2) was also grown in treated (alkaline treatment) and untreated sludge and in industrial wastewater (starch and slaughterhouse wastewater). All samples were autoclaved again and inoculated after cooling with 3% (*v*/*v*) *S. meliloti* inoculum.

For all rhizobial strains, the cell counts were performed on agar plates using YMA (yeast mannitol agar) with Congo red (0.25%) after appropriate serial dilution of samples with salt solution (NaCl 0.85%). Dry weight of the cells was estimated by centrifugation of the fermented broth at 7,650×*g* for 15 min followed by drying of the sedimented cells at 105°C to a constant weight. PHB was extracted as described by Comeau et al. [11] and measured with a Gas Chromatograph (Varian CP 3800) and Capillary column Zebron ZB-5 (30 m× 0.25 mm×0.25 μm) was used. The poly-3-hydroxybutyric acid (Aldrich Chemical Company, Inc) was used as a standard.

Results

Characterization of Sludge and Wastewaters

The characteristics of secondary sludge and industrial wastewaters (starch and slaughterhouse wastewater) are presented in Table 1. The starch wastewater had higher

Table 1 Characteristics of secondary sludge and industrial wastewaters used in the experiment; means of two replicates.

Parameters	Secondary sludge	Starch wastewater	Slaughterhouse wastewater
pH	5.60	3.81	6.90
Total suspended solid (%)	1	nd	nd
Total organic carbon (mg l^{-1})	4,044	5,183	1,346
Total nitrogen (mg l^{-1})	525	889	133
Total phosphorus (mg l^{-1})	208	224	20
C/N	7.70	5.83	10.12

nd Not determined

concentrations of total organic carbon (5,183 mg l^{-1}), total nitrogen (889 mg l^{-1}), and total phosphorus (224 mg l^{-1}) than those of the secondary sludge and slaughterhouse wastewater. However, the highest C/N ratio was observed in the case of slaughterhouse wastewater (C/N=10.12).

Sludge as Medium for Rhizobial Growth

Results of rhizobial growth in sludge and in standard medium are presented in Fig. 1. Final cell concentration, generation time and PHB yield (after 72 h of growth) are presented in Table 2. In secondary sludge, for all strains, the generation time was definitely higher than that obtained on standard medium. Generally, values ranged from 7.81 (for *S. meliloti*) to 16 (for *R. leg*. bv. *phaseolus*) and from 5 (for *S. meliloti*) to 8.27 h (for *R. leg*. bv. *viciae*) in sludge and in standard medium, respectively. Except for *S. meliloti,* the other strains showed an adaptation period varying from 15 (in the case of *R. leg.* bv *viciae* and *R. leg*. bv. *trifolii*) to 30 h (in the case of *R. leg*. bv. *phaseolus*) while growing in sludge. Concerning, the cell concentration, after 72 h of growth, values varied depending on the strain used. Highest values (around 3×10^9 cfu ml^{-1}) were obtained for *S. meliloti* in sludge similarly to standard medium. PHB yields in standard medium varied for the different strains, with values ranging between 27.41% (for *R. leg*. bv. *trifolii*) and 40.49% (for *R. leg*. bv.

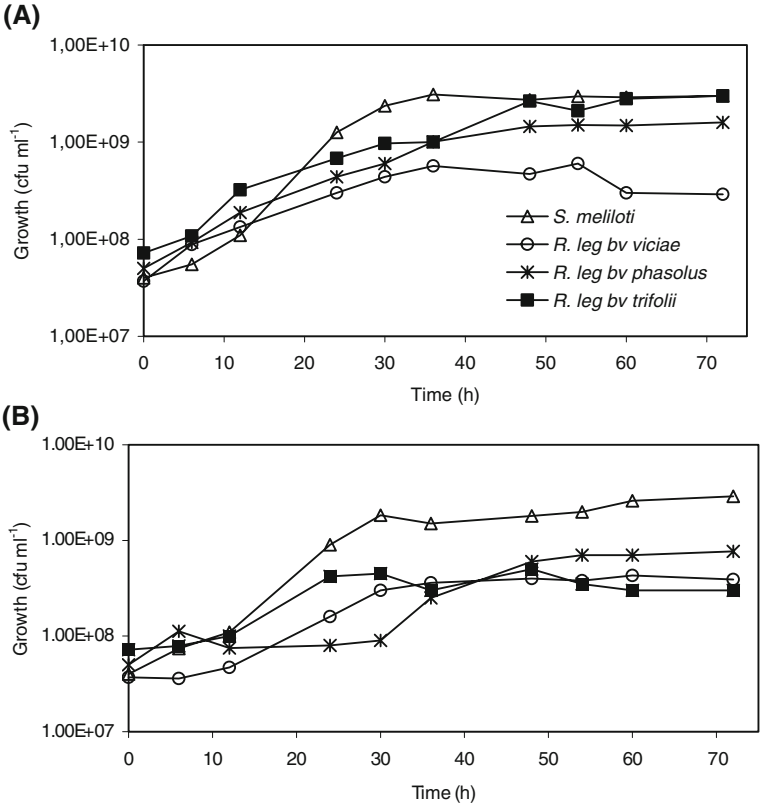

Fig. 1 Growth of rhizobial strains in standard medium (**a**) and in secondary sludge (**b**); means of two replicates

Table 2 Results of rhizobial growth in standard medium and in secondary sludge; means of two replicates.

	Standard medium			Secondary sludge		
	X_f (cfu ml^{-1})	t_g (h)	PHB (% *w*/*w*)	X_f (cfu ml^{-1})	t_g (h)	PHB (% *w*/*w*)
S. meliloti	3.0×10^9	5.00	32.14	2.9×10^9	7.81	3.70
R. leguminosarum bv *viciae*	2.9×10^8	8.27	32.70	3.9×10^8	9.21	3.56
R. leguminosarum bv *phaseoli*	1.6×10^9	8	40.49	7.7×10^8	16	3.60
R. leguminosarum bv *trifolii*	3×10^9	7.75	27.41	3×10^8	9.71	3.55

X_f Final cell count (cfu ml^{-1}) after 72 h of growth, t_g generation time (h), *PHB* yield of PHB (% *w*/*w*) produced after 72 h of growth.

phaseoli). However, in sludge PHB, production did not exceed 3.7% for all strains after 72 h of growth.

Effect of Sludge Alkaline Treatment on *S. meliloti* Growth Performances

Table 3 and Fig. 2 summarize growth characteristics of *S. meliloti* in treated and untreated sludge (with 1 and 1.5% TSS concentrations). With untreated sludge, the strain showed a long adaptation period. The generation time was about 7.81 and 10.56 h for 1 and 1.5% TSS, respectively. Moreover, at 1.5% TSS, the cell count did not exceed a maximum of 3.4×10^8 cfu ml^{-1}(Fig. 2) which was lower compared to value obtained with 1% TSS (1.57×10^8 cfu ml^{-1}). However, the alkaline treatment reduced the generation time and enhanced the maximum cell count for both treated sludge samples (1 and 1.5% TSS). For example, at 1.5% TSS, the cell count reached a maximum of 4.8×10^9 cfu ml^{-1} and the generation time was about 3.16 h. Concerning the PHB produced by *S. meliloti*, the highest PHB yield obtained was 7.27% *w*/*w* (corresponding to cell count of 1.4×10^9 cfu ml^{-1}) in untreated sludge with 1% TSS concentration after 60 h of cultivation. Increasing the TSS concentration to 1.5% reduced the maximum PHB yield to 3.64% *w*/*w*. However, this value was obtained at 36 h with a cell count of 2×10^8 cfu ml^{-1}. Alkaline treatment of 1% TSS sludge reduced the time for reaching the highest PHB concentration (6.39% *w*/*w* obtained at 36 h) without increasing significantly the cell count at this time (3×10^9 cfu ml^{-1}). However, this value was slightly lower than that obtained without treatment (7.27%). At 1.5% TSS, alkaline pre-treatment did not improve the PHB accumulation.

Table 3 Growth characteristics of *S. meliloti* in treated and untreated secondary sludge; means of two replicates.

	TSS 1%				TSS 1.5%			
	X_m (cfu ml^{-1})	t_g (h)	X_t (cfu ml^{-1})	PHB_m (% *w*/*w*)	X_m (cfu ml^{-1})	t_g (h)	X_t (cfu ml^{-1})	PHB_m (% *w*/*w*)
Untreated	1.57×10^9	7.81	1.4×10^9	7.27	3.4×10^8	10.56	2.0×10^8	3.64
Treated sludge	3.7×10^9	3.17	3.0×10^9	6.39	4.8×10^9	3.16	4.3×10^9	1.96

X_m maximum cell count (cfu ml^{-1}) obtained during growth, X_t cell count (cfu ml^{-1}) corresponding to the maximum PHB yield, t_g generation time (h), PHB_m maximum yield of PHB (% w/w) produced during growth

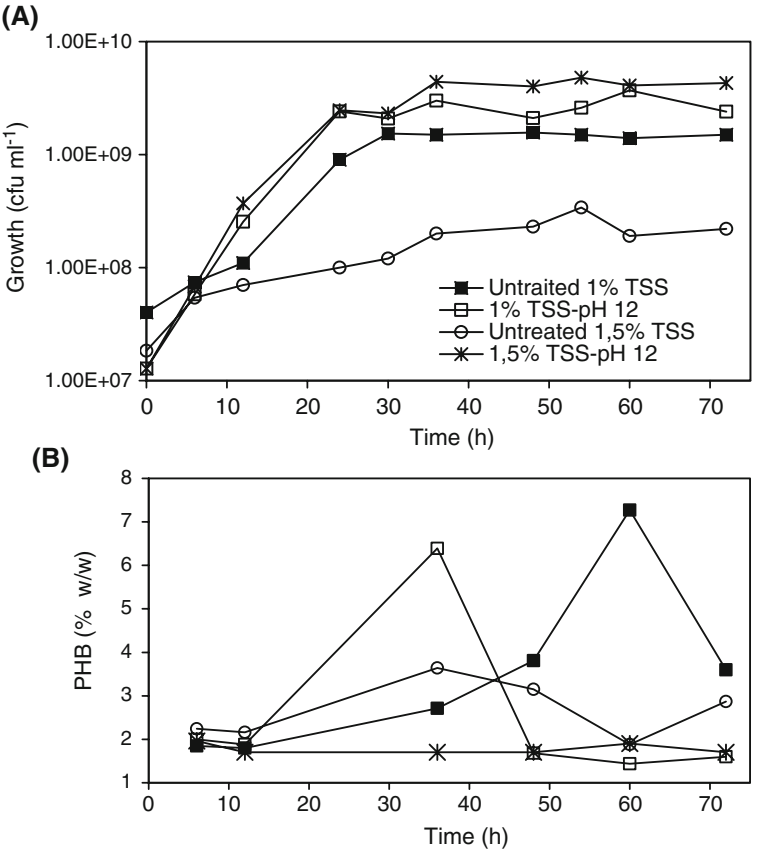

Fig. 2 Growth (**a**) and PHB production (**b**) of *S. meliloti* grown in treated and untreated secondary sludge with different TSS concentrations; means of two replicates

Industrial Wastewater as Growth Medium for *S. meliloti*

Results presented in Fig. 3 showed growth and PHB accumulation of *S. meliloti* cultivated in industrial wastewaters. The strain grew well in slaughterhouse wastewater; the maximum cell count and the generation time were, respectively, about 5.7×10^8 cfu ml^{-1} and 3.9 h. These values were different from those obtained while growing the strain on starch wastewater (3.7×10^9 cfu ml^{-1} and 7.12 h). During growth, PHB production fluctuated and did not exceed 10.7% *w/w* (obtained after 36 h and corresponding to a cell count of 3.3×10^8 cfu ml^{-1}) and 3% (obtained after 36 h and corresponding to a cell count of 1.24×10^9 cfu ml^{-1}) in slaughterhouse wastewater and in starch wastewater, respectively.

Discussion

All species of rhizobia tested in this work (*S. meliloti*, *R. leg.* bv *viciae*, *R. leg.* bv *phaseoli*, and *R. leg.* bv *trifolii*) were able to grow and utilize nutrient contained in sludge and in wastewater. Growth parameters were affected by the nature of the used medium. These results confirmed these obtained by Ben Rebah et al. [12–16]. Hence, it was demonstrated

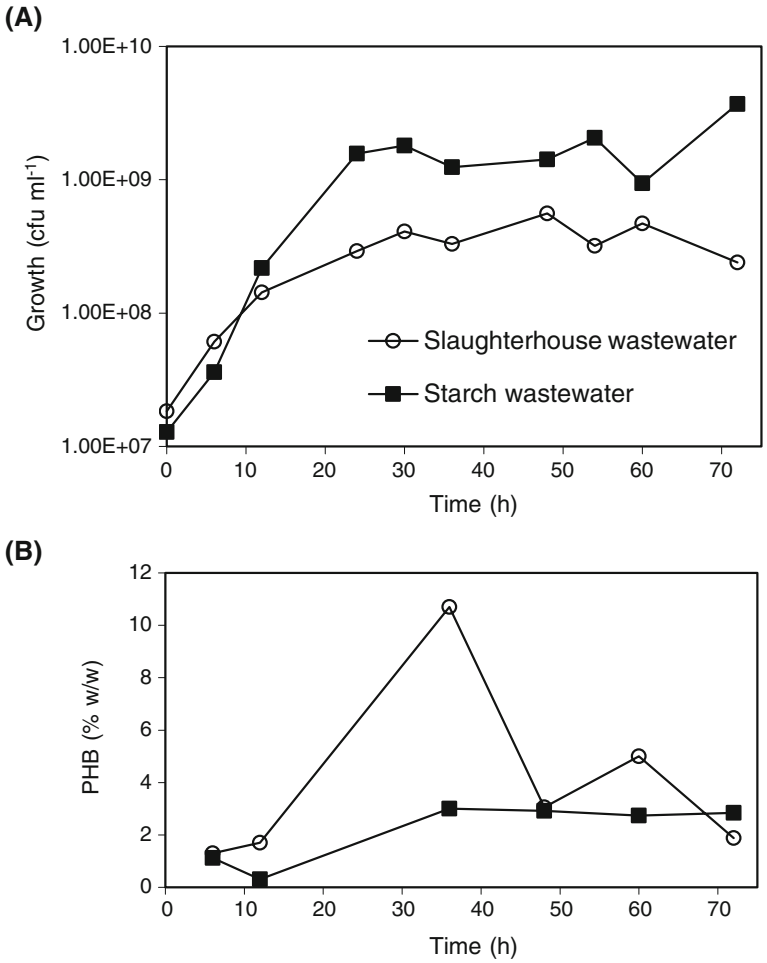

Fig. 3 Growth (**a**) and PHB production (**b**) of *S. meliloti* grown in industrial wastewater; means of two replicates

that wastewater sludge contains sufficient carbon, nitrogen, phosphorus, and micronutrients to sustain fast- and slow-growing rhizobia. Therefore, industrial wastewater and sludge can be used as an approach to substantially reduce the pollution load and simultaneously produce value-added product such as biofertilizer and PHB. PHB is carbon storage polymer widely distributed among fast- and slow-growing rhizobia and nodule bacteriodes [17].

All rhizobial strains can accumulate 27 to 40% PHB in standard medium, which is about ten times the amount produced in wastewater sludge (maximum 3.7% *w*/*w*). The better growth and PHB accumulation in standard medium may be explained by a better availability of the carbon source compared to sludge. There is also variability in PHB production among species in standard medium, which may be due to intrinsic species/strains characteristics. It was demonstrated that rhizobia can accumulate PHB up to 70% (*w*/*w*) in the case of *Rhizobium spp* 2426 selected by Mercan et al. [18] and cultivated in yeast extract containing L-cysteine. We can suppose that strains growing in limiting amounts of carbohydrate produced considerably less polymers due to its utilization as

nutrient. Therefore, in the case of sludge, the major quantity of the available carbon supposed to be used for bacterial culture. Moreover, the lower quantity of PHB accumulated by bacteria is breakdown due to its utilization during growth, as a response to the exhaustion of available nutrients in sludge. Sludge pre-treatment however, could increase the availability of carbon and, consequently, the PHB content.

In slaughterhouse, *S. meliloti* grows less and accumulates more PHB than in starch wastewater. In all cases, after a period of growth, a reduction of PHB in cell was observed. This could be explained by the exhaustion of the carbon source. To overcome this problem, the cells should be harvested before they enter the stationary phase. Fed-batch culture can also be used to enhance the cell PHB content. Generally, the nature of the carbon source and nitrogen sources affected both biomass formation and biopolymer production by rhizobia. In other studies, it was demonstrated that growth and PHB accumulation were limited while growing *S. meliloti* (strain Su47 and M5N1) in a nitrogen-deprived medium [19]. Moreover, the assimilation of nitrate by *S. meliloti* gave lower PHB yields [20]. This underlines the importance of nitrogen in the synthesis of biopolymers. Therefore, it is very important to determine the nature of nitrogen in sludge and in wastewater (Nitrate, NTK, NH_4-N). However, according to Table 1, the C/N may have an impact on PHB accumulation. Hence, the highest PHB was obtained while growing *S. meliloti* in slaughterhouse wastewater, which indicated a C/N ratio (about 10) considerably higher compared to sludge and starch wastewater.

Because rhizobia accumulate biopolymers, the potential of growing rhizobia in wastewater can lower the cost of PHB production and wastewater treatment. However, more investigations are needed to examine factors affecting PHB production from wastewater and in sludge, which are considered as abundant and inexpensive substrates.

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