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Evaluating the Feasibility of Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) Co-Biopolymer Production from Rice Wastewater by *Azohydromonas lata*

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Abstract

Background and objective: Biopolymers are environmental friendly, non-toxic renewable alternatives for conventional synthetic polymers. Rice wastewater contains high biochemical and chemical oxygen demands and organic contents mainly in form of starch which can cause serious environmental problems, while, it can be used as a potentially low-cost substrate for biopolymer production. The objective of the current study was to investigate the ability of *Azohydromonas lata* to produce poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P3HB-*co*-P3HV) from rice wastewater in a batch system.

Material and methods: *Aspergillus niger* was first used to hydrolyze the starch content of rice wastewater to fermentable soluble sugars. Then, the bacterium *Azohydromonas lata* was cultured in hydrolyzed wastewater at various C: N: P ratios to produce biopolymers. So, effects of different nitrogen and carbon sources on P (3HB) and P (3HV) contents at a C: N: P ratio of 100:4:1 were assessed.

Results and conclusion: This study showed that *Azohydromonas lata* was able to produce poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P3HB-*co*-P3HV) from rice wastewater in the presence of simple carbon sources and under limited nutrient conditions, especially phosphorus. The highest content of P (3HB) was achieved when ammonium sulphate was used as nitrogen source at a C: N: P ratio of 100:4:1. The highest recorded cell dry mass and biopolymer concentration were 4.64 and 2.8 g l^{-1} respectively, at a P(3HB) content in biomass of 60% w w^{-1}. Results indicated that phosphorus and nitrogen limitations could significantly affect P (3HB) production. In general, rice wastewater is a potential alternative for carbon sources such as glucose and maltose in polyhydroxybutyrate production.

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1. Introduction

The massive production of petroleum-based plastic wastes has caused serious environmental concerns throughout the world. Plastics are extensively used due to their excellent mechanical and thermal properties while their long-term presence in the environment poses several issues to the ecosystem. Plastic wastes are deposited in landfills, burned or recycled. Degradation rate of the plastics in landfills is tremendously low. Burning of plastics generates highly toxic by-products, including persistent organic pollutants and dioxin [1,2].

Recycling of plastic is a time-consuming process and needs sorting of the materials. Furthermore, properties of the plastic materials may undergo some alterations [3]. The environmental risks associated with microplastics have been

the major points of recent literatures due to their potential to harm biota, affecting gastrointestinal tract, and causing starvation and death [4,5]. The potentially ecological risk assessment and spatial distribution of atmospheric microplastics were investigated by Liu et al. [6]. Moreover, Chen et al. [7] reported microplastics as emerging pollutants in marine environments. They integrated chemical analyses and in vitro bioassays to detect dioxin-like effects of microplastics. Necessity to manage problems linked to the plastic waste disposal and protect limited oil resources has encouraged the development of natural polymers. These biopolymers are environmentally friendly, non-toxic, and renewable, compared to conventional synthetic polymers. Polyhydroxyalkanoates (PHAs) are degradable polymers that are produced by microorganisms. These chemicals are degraded into water and CO_2 under aerobic conditions and into methane under anaerobic conditions using microorganisms living in soil, sea, lake, wastewater and sludge [8-10]. The PHAs are stored in various micro-organisms in the form of carbon and energy when essential sources such as nitrogen, phosphorous, sulphur, oxygen, and magnesium are limited and when carbon is available in abundance [11]. These polymers are characterized by biodegradability, non-toxicity and compatibility with the environment and can compete with oil-derived plastics [12,13].

Azohydromonas (*A.*) *lata*, formerly *Alcaligenes lata*, is the most spread bacteria, which can synthesize intracellular polyhydroxybutyrate (PHB) through utilizing simple carbon sources [14]. The bacteria is considered as a potent producer of PHB at high concentrations due to its rapid growth and ability to store biopolymers independent on growth or non-growth phases [15]. Wastewaters contain a wide variety of nutrients that can be used for the production of such environmentally friendly materials such as bioplastics. Furthermore, wastewaters can serve as cheap sources of nitrogen and carbon to produce value-added products through fermentation processes and thus decrease production costs [16]. This technology also can reduce the concentration of nutrients in wastewaters and hence decrease algal growth and destruction of the aquatic ecosystems [17]. Domestic and urban wastewaters, which generally contain degradable materials and nutrients (e.g. phosphorous, carbon and nitrogen compounds) with no complicated compositions, can be appropriate sources for the growth of biopolymer-producing microorganisms. Relatively, PHA production from different types of wastewaters, including oil mill waster [18], municipal wastewater [19], brewery wastewater [20], synthetic oily bilge water [21], and wet oxidation liquors [22] have been investigated.

Rice is ranked as the most important main food in addition to cereal and wheat in Asia, particularly in Iran, where the consumption rate is 110 g per capita day^{-1} [23]. In residential, industrial and commercial kitchens, when rice is drained through colanders, large quantities of wastewater flow into the sewerage pipes. Such wastewater has high BOD (biochemical oxygen demand), COD (chemical oxygen demand), and organic materials (mostly starch), potentially damaging the environment [24-26]. If these wastewaters are not treated properly, they may contaminate water systems and cause unpleasant odours. Hence, the present study was conducted to produce environmentally friendly bioplastics from rice wastewater as a cost-effective carbon source for the first time. Furthermore, this study investigated the production of co-biopolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P3HB-*co*-P3HV) from rice wastewater using structurally unrelated carbon sources such as fructose and glucose without adding precursor substrates structurally related to 3HV and determined the optimal C:N:P ratio for the production of high quantities of biopolymers at low costs.

2. Materials and methods

2.1. Rice wastewater properties and hydrolysis conditions

Rice wastewater was collected from the canteen in Faculty of Natural Resources and Marine Sciences, Tarbiat Modares University, Noor, Iran. Wastewater physic-chemical parameters were assessed using standard methods (Table 1). Since the wastewater was rich in starch, it was hydrolysed to monosaccharide and simple sugars to support the growth of bacteria in the fermentation process. Therefore, *Aspergillus niger* was used to hydrolyse the starch content of rice wastewater. The fungus produces alpha-amylase (EC 3.2.1.1) enzyme, which results in the breakdown of starch granules contain amylose and amylopectin into dextrin [27].

Table 1. Physicochemical properties of the rice wastewater

Parameters	Value	Unit
pH	7.7	-
Biochemical oxygen demand	18000	mg l^{-1}
Chemical oxygen demand	20000	mg l^{-1}
Total suspended solids	5000	mg l^{-1}
Volatile suspended solids	3370	mg l^{-1}
Fixed suspended solids	1630	mg l^{-1}
Soluble sugar before hydrolysis	7000	mg l^{-1}
Soluble sugar after hydrolysis	16000	mg l^{-1}
PO_4^{3-}-P	24	mg l^{-1}
NH_4^+-N	343	mg l^{-1}

2.2. Fungal hydrolysis of rice wastewater

Freeze-dried *A. niger* DSMZ 823 was provided by the German culture collection (Deutsch Sammlung von Mikro-organismen und Zellkulturen). The fungi culture media included 50 g l^{-1} sucrose, 4.5 g l^{-1} $(NH_4)_2SO_4$, 1.5 g l^{-1} KH_2PO_4, 1 g l^{-1} K_2HPO_4, 2.5 g l^{-1} $MgSO_4$ and 1 g l^{-1} yeast extract. All chemicals were purchased from Merck Company. The fungus was then inoculated into the rice

wastewater and the culture was incubated at 30°C for 5 days at pH 5.5 using shaking incubator (Lovibond, Germany). The cell culture was centrifuged at 2605 g and the supernatant was collected to be used as bacterial growth media [28].

2.3. Bacterial cell culture

A pure bacterial strain of *A. lata* DSMZ 1123 was purchased from DSMZ, Germany, in freeze-dried form. The bacteria were instantly transferred to peptone yeast extract broth and then to the inoculum media to prepare the most active cell mass for the inoculation into the main fermentation media. When the cellular concentration reached 1 g l^{-1} in the inoculation medium, cells were transferred to the experimental media (rice wastewater). The culture media consisted of 10 g l^{-1} glucose, 0.5 g l^{-1} $NaHCO_3$, 2.9 g l^{-1} Na_2HPO_4, 2.3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4.7H_2O$, 1 g l^{-1} NH_4CL, 0.01 g l^{-1} $CaCl_2.2H_2O$, 0.05 g l^{-1} NH_4Fe(III) citrate, as well as trace elements including 100 g l^{-1} $ZnSO_4.7H_2O$, 300 g l^{-1} H_3BO_3, 200 g l^{-1} $CoCL_2.6H_2O$, 6 g l^{-1} $CuSO_4$, 20 g l^{-1} $N_iCl_2.6H_2O$, 30 g l^{-1} $Na_2MoO_4.2H_2O$ and 100 g l^{-1} $MnCl_2.2H_2O$ [29].

2.4 Fermentation process and sampling

The hydrolysed wastewater, containing soluble sugars, was used instead of glucose for the bacterial cell cultivation. The fermentation experiments were carried out in Erlenmeyer flasks. The fermentation medium was inoculated with 3% of seed cultures and incubated at 30°C using incubator shaker (Lovibond, Germany). Samples were collected every 24 h for 6 consecutive days to measure the cell growth, sugar consumption, biopolymer production and nitrogen ions (NH_4^+-N) and phosphorous (PO_4^{3-}-P) concentrations. To determine the concentration of sugar in the wastewater and the amount of sugar used for the cell growth, the concentrations of reducing sugars were measured using DNS method [28].

It is essential to determine the cell dry mass (CDM) in microbial culture for designing, controlling, optimizing, and scale increasing purposes. Therefore, 5 ml of the bacterial suspension were collected from the fermentation medium and centrifuged at 2605g for 20 min. The upper liquid was removed and the biomass was fully dried at 70°C using oven to reach a fixed weight. The amount of nitrogen in the culture medium was measured via Kjeldahl (Kjeldahl Distillation Unit, VELP UDK 142, Italy) and AOAC methods [28,30]. The phosphorous content of the fermentation medium was measured by the vanadate-molybdate ammonium method using spectrophotometer (UNICO SQ-4802, USA).

2.5. PHAs measurement using gas chromatography (GC)

In general, 5 ml of the sample were collected from the culture media and centrifuged at 2605 ×g for 20 min under sterile conditions. After removing the upper liquid, 2 ml of acidic methanol (3% sulfuric acid) and 2 ml of chloroform were added to the extracted cells in test tubes tightly closed with Teflon cap and kept in a water bath for 3.5 h at 100°C [29]. After cooling, 1 ml of deionized water was added to each tube and agitated for 5 min. After 60 min, the following phases were observed at upper, middle and lower positions as water and methanol, microbial residues, and methyl ester hydroxyalkanoate, respectively. The lower phase was separated for GC analysis. Totally, 20 µl of methyl benzoate as internal standard were added to the samples before GC injection to measure PHA concentration [28].

The GC apparatus (Dani GC 1000, Italy) equipped with flame ionization detector and a capillary column (BP20, SGE, Australia) with 0.33 mm of internal diameter and 25 m of length was used to detect PHAs in the splitless mode. The initial oven temperature was set at 100°C; after 1 min, the temperature was augmented at a rate of 8°C min^{-1} to 180°C and kept at this temperature for 5 min. Then, temperature increased to 200°C at a rate of 10°C min^{-1}. The injector and flame ionization detector temperatures were set at 250 and 280°C, respectively. Helium was used as the carrier gas [28].

2.6. Yield and productivity calculations

The cell production yield was calculated according to the following equation:

$$Y_{X/S} = -\frac{\Delta X}{\Delta Y} = -\frac{X_f - X_i}{S_f - S_i} \quad (1)$$

Where X_f and X_i are the final and initial CDM and S_i and S_f are sugar concentrations at the beginning and end of the culture process, respectively. The biopolymer production yield was calculated as follows:

$$Y_{P/S} = -\frac{P_f - P_i}{S_f - S_i} \quad (2)$$

Where P_i and P_f are the amount of produced biopolymer at the beginning and end of the growth period, respectively. Volumetric productivity is an important factor in industrial processes that helps to determine the required time for the optimal production. It is calculated by dividing the quantity of produced biopolymer to the culture time period, represented in g $l^{-1}h^{-1}$ [28]:

$$Q_p = \frac{\Delta P}{\Delta T} = \frac{P_f - P_i}{t} \quad (3)$$

2.7. Design of experiments

A brief description of the experiments is summarized in Table 2. In experiments 1 to 5, various concentrations of ammonium chloride, as a nitrogen source, were used while the carbon and phosphorous were constant. In experiments 6 and 7, a fixed ratio of C: N=25 was used while the phosphorous varied. In experiments 8 to 10 with an optimal ratio of C: N: P as 100:4:1, the following carbon resources were used; wastewater, wastewater plus acetate, and wastewater plus butyrate. In experiments 11 to 14 with the optimal C: N: P= 100:4:1, the impact of various nitrogen

resources, including ammonium sulphate, ammonium nitrate, ammonium chloride and urea was examined. Ultimately, in experiments 15 to 18 with the optimal ratio of C: N: P= 100:4:1, the effects of carbon sources such as fructose, glucose, sucrose, and maltose on the microorganism growth and biopolymer production were evaluated.

2.8. Statistical analysis

All samples were tested in triplicate to verify reproducibility of the results. One-way analysis of variance was used to show statistically significant differences between the means of three independent variables, including carbon, nitrogen, and phosphorus. Results were statistically significant when $P \leq 0.05$. Statistical analysis was carried out using SPSS Software version 22 (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Effect of nitrogen as the limiting factor on cell growth and biopolymer production

Nutrients restriction can stimulate PHAs production, especially restriction in nitrogen, which is an important factor for controlling cell production and growth. According to the literatures [31], when nitrogen in the culture media of *A. lata* is limited, the P(3HB) content increases significantly. In the current study, ammonium chloride was used as nitrogen source and effects of various concentrations of nitrogen on growth of *A. lata* and biopolymer production were investigated, while C: P ratio was fixed at 100:10. In this regard, various C:N:P ratios of 100:1:10, 100:2:10, 100:4:10, 100:6:10 and 100:8:10 were considered and their effects on the microorganism growth and biopolymer production were assessed using rice wastewater as carbon source. The results are summarized in Table 3. As C: N ratio decreased from 100:1 to 100:4, the biopolymer production increased from 0.75 to 1.37 g l^{-1} within 96 h. However, a decrease was detected in biopolymer production as the C: N ratio exceeded 100:4. Several studies investigated the influence of C: N ratio on biopolymer production and microorganism growth. Cui et al. [32] assessed the effect of C N^{-1} ratio on the simultaneous production of PHA and extracellular polymeric substances by *Haloferax mediterranei*. The maximum PHA content was detected at C N^{-1}=35 under limited nitrogen conditions while the content was remained stable at a C N^{-1} ratio lower than 15. In another study, Mokhtarani et al. [31] showed that an increase in C:N ratio improved the biopolymer production. However, there was no significant effects on PHA production at high C:N ratios, which might be due to the lack of nutrients such as nitrogen in the culture media that can decrease the cell activity and biopolymer production. In the current study, insignificant differences in CDM were recorded at different C:N ratios. The highest CDM (2.39 g l^{-1}) was obtained at C:N= 100:6 (Table 3).

Table 2. Design of the experiments co-biopolymer production from rice wastewater by *Azohydromonas lata*

Run	C:N:P	C (g l^{-1})	N-NH_4^+ (g l^{-1})	NH_4Cl (g l^{-1})	PO_4^{3-}-P (g l^{-1})	$Na_2HPO_4.2H_2O$ (g l^{-1})	KH_2PO_4 (g l^{-1})
1	100:1:10	31.5	0.315	1.2	3.51	1.75	1.390
2	100:2:10	31.5	0.63	2.4	3.51	1.75	1.390
3	100:4:10	31.5	1.26	4.8	3.51	1.75	1.390
4	100:6:10	31.5	1.89	7.22	3.51	1.75	1.390
5	100:8:10	31.5	2.52	9.63	3.51	1.75	1.390
6	100:4:1	31.5	1.26	4.81	0.32	0.18	0.140
7	100:4:20	31.5	1.26	4.81	6.30	3.51	2.780
8	100:4:1	21.7	0.868	3.317	0.22	0.12	0.095
9	100:4:1	23	0.92	3.51	0.23	0.13	0.100
10	100:4:1	25.5	1.02	3.89	0.26	0.14	0.110
11	100:4:1	21	0.84	3.96	0.21	0.12	0.093
12	100:4:1	21	0.84	4.8	0.21	0.12	0.093
13	100:4:1	21	0.84	3.21	0.21	0.12	0.093
14	100:4:1	21	0.84	3.6	0.21	0.12	0.093
15	100:4:1	20	0.8	3.05	0.20	0.11	0.088
16	100:4:1	20	0.8	3.05	0.20	0.11	0.088
17	100:4:1	21	0.84	3.21	0.21	0.12	0.090
18	100:4:1	21	0.84	3.21	0.21	0.12	0.090

Table 3. Biopolymer production yield, cell dry mass and volumetric productivity values of the hydrolyzed rice wastewater at various C: N ratios and C: P=10

C:N	P(3HB) (g l^{-1})	Cell dry mass (g l^{-1})	P(3HB) (%)	Sugar consumption (%)	Production yield (g g^{-1})	Cell yield (g g^{-1})	Volumetric productivity (g l^{-1} h^{-1})	Max production time (h)
100:1	0.75 ± 0.04	2.37 ± 0.12	32.85 ± 1.60	22.00 ± 1.10	0.17 ± 0.009	0.54 ± 0.030	0.011 ± 0.0006	72.00
100:2	0.86 ± 0.04	2.30 ± 0.12	39.60 ± 1.98	25.00 ± 1.20	0.12 ± 0.006	0.28 ± 0.014	0.013 ± 0.0007	72.00
100:4	1.37 ± 0.07	2.31 ± 0.12	60.00 ± 3.00	26.00 ± 1.30	0.16 ± 0.008	0.26 ± 0.013	0.015 ± 0.0007	96.00
100:6	1.13 ± 0.06	2.22 ± 0.11	51.45 ± 2.50	27.00 ± 1.30	0.17 ± 0.008	0.25 ± 0.013	0.016 ± 0.0008	72.00
100:8	0.90 ± 0.04	2.39 ± 0.12	36.26 ± 1.81	28.00 ± 1.40	0.15 ± 0.008	0.26 ± 0.013	0.012 ± 0.0006	72.00

- Ammonium chloride was used as nitrogen source
-The initial sugar concentration was 31 g l^{-1}
-The values are based on mean ± SD (standard deviation) with three replications

Consequently, the optimal C:N ratio for the highest CDM included no correlation with that for the highest PHA production. With the decrease of C: N ratios, the CDM was increased while the biopolymer production reduced. The highest CDM and the lowest biopolymer production were observed at C:N= 100:8. In a similar study, Amini et al. [33] reported that the optimal ratio for the highest cell dry weight was different from the optimal ratio for the highest PHA production. The cell dry weight decreased with an increase in C:N ratio and the highest quantity of 2.7 g l^{-1} was obtained at C:N=100:6. Moreover, the PHA accumulation was enhanced with the increase of C: N ratio and reached to 1.11 g l^{-1} at C:N=100:2. These results support the findings of the current study that an increase in C: N ratio up to a particular value enhances the biopolymer production and further increase in C:N ratio prevents the biopolymer production. In most studies, carbon sources were partially consumed by the microorganisms and nearly 25% of the sugar was consumed. This is due to uncontrollable pH levels in batch systems. Other researchers have also verified that acidification of the environment and decrease in pH values restrict the bacterial growth, thereby, incomplete consumption of carbon sources occurs [34].

3.2. Effect of phosphorus as limiting factor on cell growth and biopolymer production

In the present study, the effect of various concentrations of phosphorous on cell growth and product formation was investigated. For this purpose, various C:N:P ratios, including 100:4:1, 100:4:10 and 100:4:20 were experimented while C: N ratio was constant at 100:4. As can be seen in Table 4, results displayed that variations in phosphorous concentration affected CDM and biopolymer production. The maximum CDM (2.85 g l^{-1}) was achieved at a C:P ratio of 100:20, while the minimum quantity of biopolymer was produced at the same ratio. The maximum P(3HB) concentration of 1.65 g l^{-1} (79.4% w w^{-1}) was achieved at a C:P ratio of 100:1, because when the C:P ratio lowered, the carbon source was depleted due to the PHA storage [35]. A similar trend for the PHA production was reported, in which, the PHA accumulation decreased to 13% when phosphorus was in excess [36]. With an increment in phosphorous concentration, the CDM increased but less biopolymer was produced. The optimal C:P ratio for the highest CDM included no correlations with that for the highest biopolymer production. According to the results, the best C:P ratio for the cell growth and biopolymer production was 100:1, using rice wastewater as the carbon source. The maximum product yield and volumetric productivity as 0.458 g g^{-1} and 0.0175 g l^{-1} h^{-1} were respectively achieved at the same C:P ratio (Table 4). At this ratio, the lowest sugar consumption and the highest biopolymer production occurred. Comparisons between the results in Tables 3 and 4 revealed that the decreased phosphorous concentration in the culture media (from C:P of 100:10 to 100:1) caused an increase in biopolymer production from 1.37 to 1.65 g l^{-1}. Therefore, the C:P ratio of 100:1 was chosen as the best ratio for the next experiments. Compared to the carbon source, only a small amount of phosphorous was needed to achieve a high biopolymer production and keep the cells active.

3.3. Effect of the rice wastewater on cell growth and biopolymer production

Three different carbon sources including rice wastewater, rice wastewater with butyric acid as well as rice wastewater with sodium acetate were utilized and their effects on P(3HB) production was evaluated. Many studies have used mixed carbon sources to produce co-biopolymers. According to Dai et al. [37], such application of mixed carbon sources led to a higher production of co-biopolymers. Table 5 shows that the maximum CDM (5 g l^{-1}) was resulted from a mixture of rice wastewater and sodium acetate, by which, 1.66 g l^{-1} (32.6% w w^{-1}) of P(3HBV) were produced, including 1.51 g l^{-1} of monomer poly(3-hydroxybutyrate) P(3HB) and 0.146 g l^{-1} (2.9% w w^{-1}) of monomer poly(3-hydroxyvalerate) P(3HV). It is noteworthy that capability of the bacterial cells to synthesize biopolymers significantly depends on carbon substrates and precursors of the target monomer units. To avoid toxicity or inhibition, the type and dose of precursors should be adjusted [38]. Raza et al. [39] reported the synthesis of P(3-HB-*co*-3-HV) using propionic acid as precursor. Another literature highlighted the propionic acid and valeric acid as precursors for 3PHV units [40], whereas the P(3HV) polyester was surprisingly synthesized in this study through media enrichment with simple carbon sources.

Table 4. Biopolymer production efficiency, cell dry mass and volumetric productivity values of the hydrolyzed rice wastewater at various C:P ratios and C:N=25

C:P	P(3HB) (g l^{-1})	Cell dry mass (g l^{-1})	P(3HB)(%)	Sugar consumption (%)	Production yield (g g^{-1})	Cell yield (g g^{-1})	Volumetric productivity (g l^{-1} h^{-1})	Max production time (h)
100:1	1.65 ±0.082	2.0±0.10	79.40 ± 3.90	12.00 ± 0.60	0.46 ± 0.020	0.68± 0.03	0.018 ± 0.0009	96.00
100:10	1.37 ± 0.070	2.3±0.11	60.00 ± 3.00	26.80 ± 1.34	0.16 ± 0.001	0.26±0.01	0.015 ± 0.0007	72.00
100:20	0.29 ±0.014	2.85±0.14	10.60 ± 0.50	23.70 ± 1.18	0.04 ± 0.002	0.81± 0.04	0.003± 0.0002	96.00

Table 5. Production yield, cell dry mass and volumetric productivity values obtained from different carbon sources at C:N:P=100:4:1

Carbon source	P(3HB) (g l^{-1})	P(3HV) (g l^{-1})	Cell dry mass (g l^{-1})	P(3HB) (%)	Sugar consumption (%)	Product yield (g g^{-1})	Cell yield (g g^{-1})	Volumetric productivity(g l^{-1} h^{-1})
•WW	1.83 ± 0.09	-	3.85 ± 0.20	44.70 ± 2.40	44.50 ± 2.22	0.25±0.013	0.54±0.020	0.019 ± 0.0009
••WW+BA	0.55 ± 0.03	-	4.25 ± 0.21	13.00 ± 0.64	41.00 ± 2.05	0.86±0.004	0.47±0.023	0.006 ± 0.0003
•••WW+AC	1.51 ± 0.07	0.1±0.007	5.00 ± 0.25	33.00 ± 1.65	48.00 ± 2.40	0.21±0.010	0.49±0.024	0.017 ± 0.0009

- Max production time was 96 h
- The initial sugar concentration was 21 g l^{-1}
- The initial concentration of butyric acid and sodium acetate was 10 g l^{-1}
- The values are based on mean ± SD (standard deviation) with three replications

• Hydrolyzed wastewater as carbon source
•• Mixture of wastewater and butyric acid as carbon source
••• Mixture of wastewater and sodium acetate as carbon source

In the present study, the addition of sodium acetate to culture media was expected to result in an increase in biopolymer production. However, an adverse effect was observed. In contrast, the use of sodium acetate at a concentration of 10 g l^{-1} resulted in an increase in CDM and a reduction in biopolymer production, compared to media containing rice wastewater alone. These results were attributed to the high concentration of sodium acetate in the culture media, which limited the achievement of desirable biopolymer production. Yu et al. [41] reported that when sodium acetate concentration was increased from 2 to 10 g l^{-1}, CDM and biopolymer production in acetate-fructose containing media decreased. However, CDM and biopolymer production increased when acetate concentration increased from 2 to 10 g l^{-1} in acetate-glucose media. The only biopolymer produced from the rice wastewater and a mixture of butyrate and rice wastewater was P (3HB) where the maximum quantity was achieved within 96 h. For the rice wastewater-butyric acid mixture, the P(3HB) production was lower due to the limiting effects of high butyrate concentrations on the cell growth. Maximum biopolymer and cell production yields of 0.25 and 0.54 g g^{-1}, respectively, were associated to the rice wastewater with no sodium acetate and butyric acid (Table 5), proving the restricting effects of the substances on the cell production yield. The highest volumetric productivity (0.019 g l^{-1} h^{-1}) was obtained with rice wastewater as the unique carbon source.

3.4. Effect of the nitrogen source on cell growth and biopolymer production

According to the obtained results, a C:N:P ratio of 100:4:1 was considered as the optimum ratio and the effects of four nitrogen sources (ammonium chloride, ammonium sulphate, ammonium nitrate and urea) on the cell growth and biopolymer production were assessed. The results are summarized in Table 6. Use of ammonium chloride as nitrogen source resulted in CDM of 3.44 g l^{-1} and P(3HB) concentration of 2.42 g l^{-1} (70% w w^{-1}) within 72 h. For ammonium nitrate, the maximum CDM and P (3HB) (50% w w^{-1}) included 4.77 and 2.4 g l^{-1}, respectively. Simultaneous increases were observed in CDM and biopolymer production, reaching their maximum values within 96 h. The maximum CDM was 4.96 g l^{-1} in the presence of urea, while P(3HB) production was 2 g l^{-1} which was attained in 120 h. For ammonium sulphate, the P(3HB) production was 2.8 g l^{-1} while the maximum CDM was 4.64 g l^{-1}. The CDM and biopolymer production increased and reached to their maximum values within 96 h (Table 6). The highest production yield (0.329 g g^{-1}) and volumetric productivity (0.033 g l^{-1} h^{-1}) were achieved using ammonium chloride. The maximum (2.8 g l^{-1}) and the minimum (2.0 g l^{-1}) amounts of biopolymer were produced using ammonium sulphate and urea, respectively.

The highest (0.033 g l^{-1} h^{-1}) and lowest (0.017 g l^{-1} h^{-1}) volumetric productivities were linked to ammonium chloride and urea, respectively. Moreover, the highest and the lowest production efficiencies as 0.329 and 0.14 g g^{-1} were obtained using ammonium chloride and urea, respectively. Regarding biopolymer to CDM ratio, 70% of the value was achieved using the former and 42% using the latter compounds. The unique generated biopolymer included P(3HB), for which, the nitrogen source was an important factor in high P(3HB) accumulation. In a research conducted by Gouda et al. [42], ammonium chloride as a nitrogen source was added to sugarcane syrup (2%). The maximum produced PHA and PHB were 40.1% and 38.4% per cell dry substance, respectively. Similar results were reported for corn syrup and wheat bran as the carbon sources with the same nitrogen source. The impact of various nitrogen sources on biopolymer production in the presence of *A. lata* and *A. eutrophus* was investigated in various media and the highest PHB level was achieved using ammonium sulphate [43]. Moreover, Lee et al. [44] studied the effects of nitrogen sources and reported that ammonium chloride was the best nitrogen source for PHBV synthesis, by which, the maximum amount of PHA (90% w w^{-1}) was produced and the minimum quantity was monitored in the presence of ammonium nitrate and sodium nitrate. In this study, results showed that more PHA was produced using ammonium chloride and ammonium sulphate than ammonium nitrate and urea. The maximum PHA (2.8 g l^{-1}) was produced in the presence of ammonium sulphate, thereby,

this nitrogen source was more appropriate for the microorganism used in the current study. Despite the reduction in CDM, the ammonium chloride seems better than ammonium sulphate in terms of intracellular biopolymer contents (70% w w^{-1}). Therefore, the least consumption of sugar (% w w^{-1}) occurred in the presence of ammonium chloride, while sugar was totally consumed in media containing ammonium sulphate.

3.5. Effect of the carbon source on cell growth and biopolymer production

The effect of different carbon sources on PHA production was investigated to choose the best condition in comparison with the wastewater carbon source. 1.52 g l^{-1} of CDM was produced in the presence of fructose and 1.447 g l^{-1} of poly-3-hydroxyvalerate (92% w w^{-1}) was obtained which was the highest amount compared with those produced in the presence of glucose, maltose and sucrose (Table 7). The CDM of 1.43 g l^{-1} and 0.82 g l^{-1} of P (3HV) (56.54% w w^{-1}) were detected when sucrose was used as carbon source. Furthermore, the maximum amount of CDM (3.26 g l^{-1}) was observed using maltose as carbon source, resulting in 0.27 g l^{-1} of PHV (9.5% w w^{-1}). However, the use of glucose resulted in CDM of 3.25 g l^{-1} which contained 0.133 g l^{-1} of PHV (4.4% w w^{-1}). The similar results were reported by Daneshi et al. [28], who declared that fructose was the only sugar consumed by *A. eutrophus* while glucose or disaccharides such as lactose, maltose, or sucrose remained unused, which depicted the high efficiency of fructose as carbon source. In another study carried out by Tabandeh and Vasheghani-Farahani [45], the use of *Ralstonia eutropha* ACM 1296 and fructose led to a cell production yield of 0.3 g g^{-1}. These researchers reported the high efficiency of fructose in PHV production. According to the results in Table 7, the unique biopolymer produced in this study was P(3HV), mostly by means of fructose and sucrose followed by maltose and glucose. However, the maximum CDM was obtained using maltose and glucose. The maximum time required to reach the highest production yield was 120 h for all carbon sources, except sucrose. Considering the sugar consumption rate, the lowest value of sugar consumption in fructose media led to the highest biopolymer production, lowering the biopolymer production costs.

It should be mentioned that fructose is more water-soluble than other sugars and hence, is easily consumed by the bacteria. The reason for a lower production of biopolymer from glucose than maltose might be linked to the bacterial inability to grow in glucose-containing media. Since maltose is a disaccharide of two glucose molecules, the bacteria is less able to consume this mentioned carbon source. The comparison between the carbon sources and rice wastewater indicated that the bacteria in the current study, was capable of merely producing PHB from the rice wastewater but it produced PHV only from fructose, maltose, glucose, and sucrose. More biopolymers were produced from the rice wastewater, despite a difference in the type of the biopolymer from four carbon sources.

Table 6. Biopolymer production yield, cell dry mass and volumetric productivity values of various nitrogen resources from hydrolyzed rice wastewater at C:N:P = 100:4:1

Nitrogen source	P(3HB) (g l^{-1})	Cell dry mass (g l^{-1})	P(3HB) (%)	Sugar consumption (%)	Production yield (g g^{-1})	Cell yield (g g^{-1})	Volumetric productivity (g l^{-1} h^{-1})	Max production time (h)
Ammonium sulfate	2.80 ± 14	4.64 ± 0.23	60.00 ±0.00	73±3.65	0.20 ± 0.010	0.30 ± 0.01	0.029±0.001	96
Urea	2.00 ± 10	4.96 ± 0.25	41.80 ± 0.00	69±3.47	0.14 ± 0.007	0.33 ±0.02	0.017±0.001	120
Ammonium nitrate	2.40 ± 12	4.77 ± 0.24	50.00 ± 0.50	70±3.52	0.17 ± 0.008	0.31 ±0.02	0.024±0.001	96
Ammonium chloride	2.40 ± 0.12	3.44 + 0.17	70.00 ± 3.50	47±2.36	0.33 ± 0.020	0.34 ±0.02	0.033±0.002	72

Table 7. Production yield, cell dry mass and volumetric productivity values of fructose, sucrose, glucose and maltose as carbon sources

Carbon source	P(3HV) (g l^{-1})	Cell dry mass (g l^{-1})	P(3HV) (%)	Sugar consumption (%)	Production yield (g g^{-1})	Cell yield (g g^{-1})	Volumetric productivity (g l^{-1} h^{-1})	Max production time (h)
Fructose	1.4 ± 0.072	1.52 ± 0.08	92.00 ± 0.60	18.0 ± 0.90	0.156 ± 0.01	0.22 ± 0.01	0.013 ± 0.00	120
Glucose	0.13 ± 0.006	3.25 ± 0.16	4.40 ± 0.22	26.0 ± 1.30	0.010 ± 0.00	0.452 ± 0.02	0.001 ± 0.00	120
Maltose	0.27 ± .013	3.26 ± 0.16	9.50 ± 0.48	30.7 ± 1.50	0.015 ± 0.00	0.14 ± 0.01	0.002 ± 0.00	120
Sucrose	0.8 ± 0.041	1.43 ± 0.071	56.00 ± 2.82	20.0 ± 1.00	0.081 ± 0.00	0.27 ± 0.01	0.014 ± 0.00	96

4. Conclusion

In general, this study presented the integrated results for the biopolymer characteristics and the optimum operational conditions to promote the growth rate of microorganisms in production of PHA biopolymer. Moreover, the results demonstrated that the hydrolysed rice wastewater was an appropriate source for the production of PHA biopolymer at a C:N:P ratio of 100:4:1. Among various nitrogen sources in this study, the best was ammonium sulphate with the maximum biopolymer production (2.8 g l^{-1}). However, the highest biopolymer productivity was resulted from C:N:P ratio of 100:4:1 as 0.033 g l^{-1} h^{-1}. Among various carbon sources, the maximum biopolymer was obtained from fructose (1.4 g l^{-1}), including P (3HV) alone. The use of mixed carbon sources such as butyric acid and sodium acetate with rice wastewater resulted in an increase in CDM and a decrease in biopolymer production. Therefore, these chemicals included no significant contributions to the higher production of biopolymer as the main objective of this study. The restriction of phosphorous in culture media had more effects on biopolymer production by *A. lata*, as compared with the nitrogen-limiting condition. A higher production rate of biopolymer (1.65 g l^{-1}) from rice wastewater by *A. lata*, showed that the rice wastewater was an appropriate alternative for production of biopolymers, compared with other carbon sources like maltose (0.27 g l^{-1}) and glucose (0.13 g l^{-1}). Despite significant progresses in increasing biopolymer production using bacterial species, serious challenges still exist which must be addressed in future studies.

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6. Conflict of interest

The authors declare no conflict of interest.

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بررسی امکان تولید زیست‌بسپار[1] پلی(۳-هیدروکسی‌بوتیرات-کو- ۳-هیدروکسی‌والرات) از پساب برنج توسط *آزوهیدروموناس لاتا*

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چکیده

سابقه و هدف: زیست‌بسپارها جایگزین تجدیدپذیر غیر سمی و سازگار با محیط زیست، غیر سمی برای بسپارهای سنتزی متداول می‌باشند. پساب برنج حاوی مقادیر زیادی ترکیبات زیست شیمیایی[2] با اکسیژن‌خواهی شیمیایی[3] زیاد و عموما به صورت نشاسته است که مشکلات زیست‌محیطی جدی را ایجاد می‌کند اما می‌تواند به عنوان سوبسترای[4] ارزان قیمت برای تولید زیست بسپار[5] استفاده شود. هدف این مطالعه بررسی توانایی باکتری *آزوهیدروموناس لاتا* برای تولید پلی(۳-هیدروکسی بوتیرات –کو-۳-هیدروکسی والرات) (P3HB-*co*-P3HV) از پساب برنج در محیط کشت ناپیوسته بود.

مواد و روش‌ها: پساب برنج ابتدا توسط قارچ *آسپرژیلوس نایجر* هیدرولیز شد تا نشاسته موجود در آن به قندهای محلول تخمیرپذیر تبدیل شود. در مرحله بعد، برای تولید زیست‌بسپار باکتری *آزوهیدروموناس لاتا* در پساب هیدرولیز شده با نسبت‌های گوناگون C: N: P کشت داده شد. علاوه بر این، تاثیر منابع مختلف کربن و نیتروژن بر مقدار پلی(۳-هیدروکسی بوتیرات) و پلی(۳-هیدروکسی والرات) در نسبت کربن: نیتروژن: فسفر ۱:۴:۱۰۰ مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: این مطالعه نشان داد که باکتری *آزوهیدروموناس لاتا* در حضور منابع ساده کربنی و محدودیت مواد مغذی به‌خصوص فسفر قادر به تولید پلی(-۳-هیدروکسی بوتیرات-کو- پلی-۳-هیدروکسی والرات) (P3HB-*co*-P3HV) از پساب برنج است. بیشترین میزان پلی(۳-هیدروکسی بوتیرات) هنگامی تولید شد که سولفات آمونیوم به عنوان منبع نیتروژن در نسبت کربن: نیتروژن: فسفر ۱:۴:۱۰۰ استفاده شد. زمانی‌که میزان پلی(۳-هیدروکسی بوتیرات) زی‌توده[6] $w\ w^{-1}$ ٪۶۰ بود بیشترین میزان توده خشک سلولی و زیست‌بسپار به ترتیب ۴/۶۴ و ۲/۸ گرم بر لیتر به‌دست آمد. نتایج نشان داد که محدودیت نیتروژن و فسفر در محیط کشت توانست به طور معنی‌داری بر تولید پلیمر زیستی پلی-۳-هیدروکسی بوتیرات تاثیر داشته باشد. به طورکلی، پساب برنج می‌تواند جایگزین بالقوه‌ای برای منابع کربنی مثل گلوکز و مالتوز در تولید پلی هیدروکسی بوتیرات باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- *آزوهیدروموناس لاتا*
- محدودیت مواد مغذی
- پلی-۳-هیدروکسی بوتیرات-کو-پلی-۳-هیدروکسی والرات
- پساب برنج

***نویسنده مسئول**

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[1] Co-biopolymer
[2] Biochemical
[3] Chemical Oxygen Demands or COD
[4] Substrate
[5] Biopolymer
[6] Biomass