Research Article

Paracoccus sp. Strain LL1 as a Single Cell Factory for the Conversion of Waste Cooking Oil to Polyhydroxyalkanoates and Carotenoids

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Abstract

Background and objective: Polyhydroxyalkanoates have drawn significant attention as alternative to petroleum-based plastics; however, their industrial production is still hindered by the costly feed materials. Co-generation of other high-value products in addition to polyhydroxyalkanoate by the same microbial strains can be helpful in alleviating overall production cost up to 50%. This study for the first time demonstrates that polyhydroxyalkanoate and astaxanthin-rich carotenoids can be co-produced by *Paracoccus* sp. LL1 using waste cooking oil as substrate.

Material and methods: The halophilic strain of *Paracoccus* sp. LL1 was grown under batch fermentation using mineral media supplemented with 1% (v v^{-1}) waste cooking oil. Different surfactants were used to improve substrate utilization. Polyhydroxyalkanoate obtained after the fermentation was characterized by fluorescent microscopy, gas chromatography, and Fourier Transform Infra-Red spectroscopy.

Results and conclusion: Oil as a substrate, led to 1.0 g l^{-1} poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) with concomitant production of 0.89 mg l^{-1} of carotenoids after 96 h. An enhancement of 2.7-folds in total cell dry mass was achieved when 0.1% (v v^{-1}) Tween-80 was used as surfactant for ease in oil metabolism. *Paracoccus* sp. LL1 has the potential to serve as a single cell factory for bioconversion of cheap substrates into high value products.

Conflict of interest: The authors declare no conflict of interest.

1. Introduction

Polyhydroxyalkanoates (PHAs) are eco-friendly bio-polyesters which have dragged attention worldwide due to their biodegradable and biocompatible nature [1,2]. These polymers can be accumulated intracellularly by a plethora of prokaryotes as carbon storage and reducing equivalent sink [3,4]. They have the potential to become a competitive alternative to synthetic polymers. However, the major factor that restricts their use is the costly production on a large-scale [5,6]. Many strategies were developed in the past few decades to overcome this obstacle. They include strain improvement (recombineering approach), biorefinery (integrative approach), cultivation optimization, and utilization of inexpensive waste biomass [7-13]. Since about 50% of the incurred cost belongs to the substrates used, it is anticipated that an effective integrative approach may curtail this cost by 45-50% [10,14]. Besides conventional feed-stocks, various agro-industrial wastes such as crude glycerol, molasses, vegetable oils, and waste cooking oil are also available in large quantities bearing the potential to be used as carbon and/or nitrogen source for PHA production [14-16]. Additionally, co-production of PHA with other value-added products is also an attractive option [10,12,17-19]. In fact, the indiscriminate exploitation of petroleum-based plastics has already displayed its negative influence on natural territory worldwide. Indeed, the growing demand for such petrochemical products is alarming and therefore research is desirable to look for its alternative, a biodegradable counterpart [4].

Ongoing advancements in integrated biorefineries for effective bioconversion of biowastes to high-value products hold the promise to bring forth a stable circular economy model [17]. The market for PHA polymers already reached >70 million USD and is expected to rise further to the tune of 93 million US-$ by 2021 [17]. In contrast, carotenoids, the red-orange pigments, especially astaxanthin (3,30-dihydroxy-β,β-carotene-4,40-dione) possess very high value equivalent to $2500-7000 kg^{-1} with an

anticipated global market value of about 1.1 billion USD by 2020. Among other carotenoids, astaxanthin is the second-most ranked since it covers around 29% of total sales. It should be noted that biotechnological astaxanthin production currently resorts to the use of microalgae, mainly *Haematococcus pluvialis* [10,11]. Astaxanthin is used as an antioxidant, aquaculture food additive (salmon and trout), pharmaceutical industries, etc. [10]. Thus, a simultaneous production of both PHA and carotenoids would potentially alleviate the cost. However, very limited studies have been conducted on this aspect. *Paracoccus* sp. LL1 is the only dark-fermentative bacteria known to synthesize astaxanthin-rich carotenoids as well as PHA [10]. The polymer extraction requires the lysis of bacterial culture by organic solvents and therefore increases the PHA production costs in any industrial fermentation set-up. In contrast, for *Paracoccus* sp. LL1, PHAs are intracellular product and carotenoids are usually membrane bound and/or secretory. Hence, this strain can be very effective for large scale production facilities for two reasons: (i) cells can be used for secretory carotenoids before harvesting the culture and (ii) the extraction solvents used for carotenoids can also be used for PHA precipitation step (i.e. effective recycling is possible). This bacterium has the ability to consume diverse carbonaceous compounds including methanol, sugars, glycerol, biowaste hydrolysates, etc.

Vegetable oils and waste cooking oil are among the cheapest carbon sources coming out from the food industries [20]. Due to their availability and cheap cost, they have been recently exploited for other biotechnological processes. However, co-production of PHA and other valuable products using oil as substrates have not been explored in detail, except for PHA and rhamnolipids [17]. In this context, the present study evaluates the potential of *Paracoccus* sp. LL1 to use vegetable oils and waste cooking oil for the co-production of PHA and carotenoids. Several surfactants were also used to enhance the oil utilization capacity of the bacterial strain. This is the first report demonstrating the waste oil bioconversion capacity of *Paracoccus* sp. LL1 into valuable products (i.e. PHA and carotenoids).

2. Materials and methods

2.1. Chemicals

All chemicals used during the study were of analytical grade (unless mentioned). Analytical standards of poly (3-hydroxybutyrate), poly (3-hydroxybutyrate-*co*-3-hydroxy-valerate) [P(3HB-*co*-3HV)]; containing 12 mol% of 3HV, β-carotene, and astaxanthin were from Sigma-Aldrich, USA and were used for PHA and carotenoid estimation.

2.2. Microorganism and culture conditions

Paracoccus sp. LL1 isolated previously in our laboratories was cultivated and maintained in Luria-Bertani agar (BD Difco™, USA) containing 10 g l^{-1} NaCl. The inoculum was prepared by transferring a single colony into 50 ml LB broth (BD Difco™, USA) containing 10 g l^{-1} NaCl in 250 ml conical flasks and kept at 200 rpm and 30 °C for 16 h. Initial tests were conducted in 500 ml conical flasks containing 100 ml of modified minimal salt (MS) medium with inoculum size of 10% v v^{-1} [21]. MS medium containing 1 g l^{-1} $(NH_4)_2SO_4$ as nitrogen source was used for bioreactor experiments [10]. Olive oil was obtained from Samchun (Republic of Korea). Castor oil was purchased from Sigma-Aldrich (USA). Waste cooking oil was collected from local market. Vegetable oils were used as substrates (sole carbon source) at a final concentration of (1% v v^{-1}) to grow *Paracoccus* sp. LL1. Three different surfactants (Tween-80, sodium dodecyl sulfate (SDS), and gum acacia) were also used at the mentioned concentration for comparative studies and to check their effect on oil metabolism.

Batch fermentation studies were conducted with a 2.5 l fermentor (KoBiotech, Republic of Korea) with an initial working volume of 1 liter. Waste cooking oil, trace elements, and salts were separately autoclaved and reconstituted under sterile condition before inoculation. MS medium containing 1% v v^{-1} waste cooking oil and 0.1% Tween-80 was inoculated (10% v v^{-1}) with *Paracoccus* sp. LL1. The agitation, dissolved oxygen, temperature, and aeration were controlled and kept at 300 rpm, >20%, 30°C, and 2.5 vvm, respectively. 2 N NaOH and 2 N HCl were used to maintain the culture at pH 7.5.

2.3. Extraction of carotenoids and PHA

Samples (10 ml each) were aseptically withdrawn after designated time intervals and were used to estimate total carotenoids as described previously [10]. The batch culture sample (1 ml) was washed with normal saline (0.85% w v^{-1} NaCl), fixed and stained with Nile blue (0.5% v v^{-1} in ethanol) before microscopic examination. The culture (100 ml) was centrifuged at 6,800 ×g for 15 min at 4°C and washed twice with saline. The freeze-dried pellets were used for estimation of cell dry mass (CDM), PHA content, and carotenoids.

2.4. Analytical methods

PHA content and oil composition were determined after trans-esterification reaction (methanolysis) of the samples using gas chromatography (GC, Agilent 6890A, Santa Clara, CA, USA) as described previously [10]. The PHA polymer extracted after 96 h of cultivation was also characterized by FT-IR (Thermo Fisher Scientific, Waltham, MA, USA) [22]. Carotenoids extracted from cells as well as culture broth were quantified using spectrophotometer and HPLC equipped with an

OptimaPak C18 column (10 × 250 mm, RS Tech, Republic of Korea). Oil consumption was measured through gravimetric separation of samples (kept in pre-weighed tubes) after solvent evaporation, as described previously [17]. Besides, total lipids were trans-esterified and the lipid content was determined using GC, as described previously [9,16]. Heptadecanoic acid methyl ester was used as an internal standard. All experiments were carried out in triplicates and the data presented are the mean values.

3. Results and discussion

PHAs are potential substitute to petroleum-based polymers and their demand in global market is rising sharply. However, in order to see these biodegradable polymers commonly in market, their production cost has to be minimized but competitive to synthetic polymers. Majority of the cost (~50%) goes towards substrates/feed. Consequently, various agro-industrial wastes are being explored for producing PHA and similar products [15,23-26]. During these times, it was realized that integrative approaches or single cell factories may help in alleviating the overall incurred cost through the synthesis of multiple products of high-value. Carotenoids are one such alternative but promising co-products that has very high-value and is in demand for their antioxidant and anticancer properties. Interestingly, we isolated and identified *Paracoccus* sp. LL1 that can accumulate both PHA as well as carotenoids using glycerol as substrate [10]. This strain is closely related to *Paracoccus marcusii* (only carotenoid producer). Thus, in the present study we focused to utilize waste cooking oil as a cheap carbon substrate for producing two bioproducts.

3.1. Effect of different oils

Initial experiments were conducted in shake flask cultures on MS medium to assess the potential of olive and castor oil as a substrate (carbon source). The optimal growth parameters for this bacterium were previously identified and were used in all experiments conducted in this study [10]. The halophilic prokaryote grew slowly on oil substrates. A maximum CDM of 695 and 790 mg l^{-1} was achieved with castor and olive oil, respectively (Fig. 1). Here, the PHA production was also very low. This reduced PHA level indicates the lower metabolism of cells, possibly because the utilization of oils directly as a substrate was difficult. Oil being larger molecule transfers slowly across the cell membrane. Keeping this in consideration, three surfactants were also tested in order to minimize the micelle formation by oil substrates. Among the three surfactants, only Tween-80 showed positive results on both CDM and PHA production. A supplementation of 0.1% Tween-80 was optimal while further higher concentration affected CDM (Fig. 1). The PHA content reached to a level of 24% of CDM equivalent to 519 mg l^{-1} at 0.1% Tween-80 supple-mentation. In contrast, acacia could not improve the yield and SDS was found to be toxic even at a lower concentration of 0.1%. Further lower concentrations of these two surfactants were also checked but it did not help in improving PHA production (data not shown). Besides this, the HV content within the polymer could not exceed beyond 2% in any of the tested conditions. Only with acacia supplementation, about 6% HV content was observed, although it seems insignificant considering the lower growth and overall P (3HB-*co*-3HV) production.
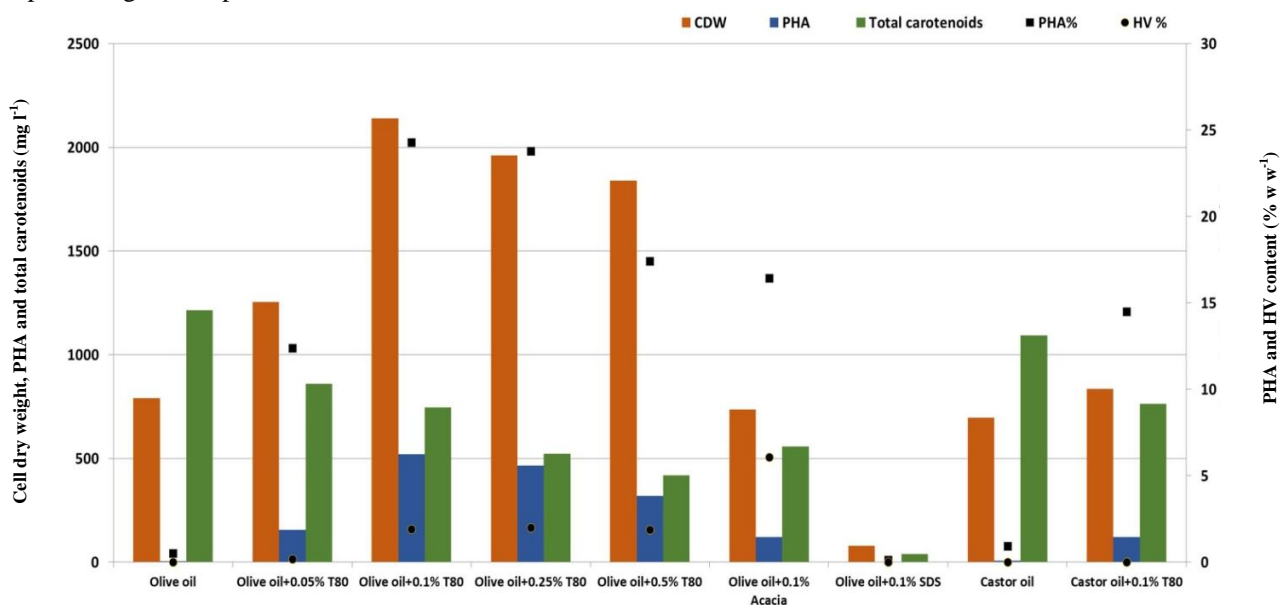

Fig. 1. Simultaneous production of P (3HB-*co*-3HV) and carotenoids by *Paracoccus* sp. LL1 on various vegetable oil substrates (1%) with or without surfactant supplementation

Interestingly, oil without surfactant led to higher carotenoid levels of more than 1 mg l⁻¹ (Fig. 1). There was a visible distinction among the color, which is possibly due to the affinity of carotenoids towards oil substrate instead of aqueous media. This indicates that with time all the cell-bound and secretory carotenoids are sequestered by the oil substrates. The total carotenoid levels were still lower than that observed with simple sugars. This could be a reflection of lower bacterial growth leading to lower total carotenoids. Overall, an optimal supplementation of 0.1% Tween-80 was identified to be suitable in order to get both products in high quantities. Compared to control, there was about 2.7 folds enhancement in the biomass and very significant increase in P (3HB-*co*-3HV) content was observed. This can be attributed to the ease in substrate metabolism in the presence of the optimal surfactant level. With Tween-80, oils were equally distributed in the culture broth. Therefore, the residual substrate itself may act as a solvent for carotenoid extraction which eventually can be separated based on the phase separation between oil and aqueous media.

3.2. Batch culture

Biotransformation of waste cooking oil to P (3HB-*co*-3HV) and carotenoids upon upscaling to 1 l fermentor on mineral media showed slight improvement over flask culture. The production levels were examined over a period of 5 days, and a steady escalation in both products was detected up to 1.5-fold enhancement in the CDM after 4 days as compared to flask culture (Fig. 2). The enhanced growth may be attributed to the fact that ample aeration and agitation declined the surface tension among the two liquid layers and provided opportunity for cells to use more oil substrates. Additionally, the HV content was quite consistent in the last 3 days and the highest P (3HB-*co*-

3HV) content reached to 30.9%. Contrary to simple sugar substrates, the P (3HB-*co*-3HV) production was delayed during the first 48 h. It can be accredited to the slow diffusion of oil substrate and the time taken by the bacteria to acclimatize with the waste cooking oil. The P (3HB-*co*-3HV) production shoots sharply during the next 2 days with a final CDM of 3.24 g l⁻¹. The intracellular P (3HB-*co*-3HV) accumulation was also observed by distinct orange fluorescence by Nile blue stain as shown in Fig. 3. After 120 h of incubation, the final biomass was still higher and consequently the carotenoid content was also enhanced. However, the level of P (3HB-*co*-3HV) declined indicating their possible utilization due to carbon source limitation. Similar observations were made on *Cupriavidus necator* and *Pseudomonas oleovorans*, although the latter can also produce surfactants. Therefore, in these studies, surfactant supplementation was not done [16,27-31]. Moreover, saponification of the oil also showed improved PHA production with *Pseudomonas* sp. Gl01 [32]. It was also suggested to explore or combine more than one waste biomass to produce PHA, particularly food industry waste rich in carbon [33]. However, it occasionally requires pretreatment before use [5,14,34]. Though PHA production observed with the *Paracoccus* sp. using various vegetable oils was not higher compared to other findings (Table 1), the synthesis of carotenoids and competitive P (3HB-*co*-3HV) yield makes this study unique. This warrants further study using this halophilic strain. The capability of *Paracoccus* sp. LL1 to metabolize many different carbon sources makes it a potential dark horse for biotechnological applications. The potential of other bacterial strains to use different oil substrate was recently reviewed, although with a member of the genus *Paracoccus*, this is the first demonstration [26,35].
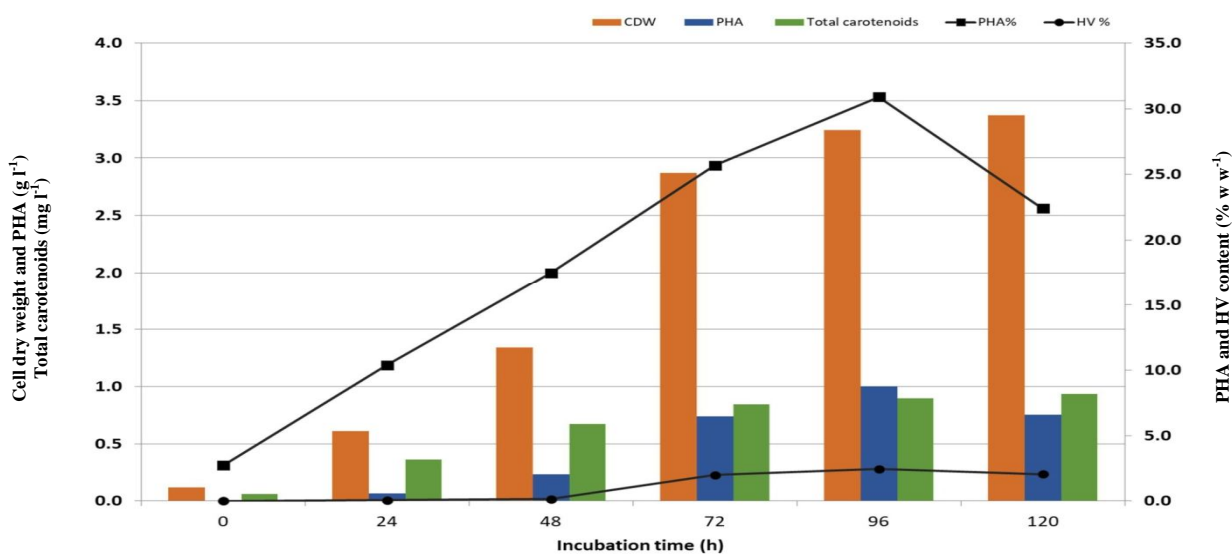
Fig. 2. Bioconversion of waste cooking oil into P (3HB-*co*-3HV) and carotenoids by *Paracoccus* sp. LL1 grown on mineral salt medium supplemented with 0.1% Tween-80

**Table 1.** Use of waste oil for polydroxyalkanoate and other high value products: A comparison

Carbon source	Bacterial strain	Culture condition	PHA-type	Co-product (if any)	Yield (CDM[a] in g l^{-1}; % PHA[b])	Yield (co-product)	References
Used cooking oil	*Burkholderia thailandensis*	Nutrient Broth; 8L Batch; 37°C; 120 h	PHB[c]	Rhamnolipids	12.6; 60.0	2.2 g l^{-1}	[17]
Palm oil (0.7%)	*Pseudomonas aeruginosa* IFO3924	Basal salt medium; 2L Batch; 30°C; 83 h	mcl-PHA	Rhamnolipids	2.2; 36.0	0.43 g l^{-1}	[18]
Cassava wastewater + waste cooking oil (2.0%)	*Pseudomonas aeruginosa* L2-1	Mineral salt medium; 0.025L Batch; 30°C; 120 h	*mcl*-PHA[d]	Rhamnolipids	4.2; 39	0.609 g l^{-1}	[14]
Waste cooking oil (2.0%)	*Pseudomonas aeruginosa* 7a	Mineral salt medium; 0.025L Batch; 30°C; 120 h	*mcl*-PHA	Rhamnolipids	6.8; 50.4	0.273 g l^{-1}	
Waste frying oil (3.0%)	*Cupriavidus necator* H16	Mineral salt medium; 1.5L Batch; 30°C; 29 h	PHB	-	25.4; 79.2	-	[35]
Used cooking oil (2.0%)	*Cupriavidus necator* DSM 428	Mineral salt medium; 2L Batch; 30°C; 50 h	PHB	-	11.6; 63	-	[31]
Coffee waste oil (1.5%)	*Ralstonia eutropha* Re2133	Minimal medium; 30°C; 72 h	*mcl*-PHA	-	0.93; 69	-	[9]
Used cooking oil (1.0%)	*Paracoccus* sp. LL1	Mineral salt medium; 1L Batch; 30°C; 96 h	PHA	Carotenoids	3.24; 30.89	0.89 mg l^{-1}	This study

[a] Cell dry mass in g l^{-1}.
[b] Polyhydroxyalkanoate content (% w w^{-1} of CDM).
[c] Poly(3-hydroxybutyrate)
[d] Medium chain length-PHA
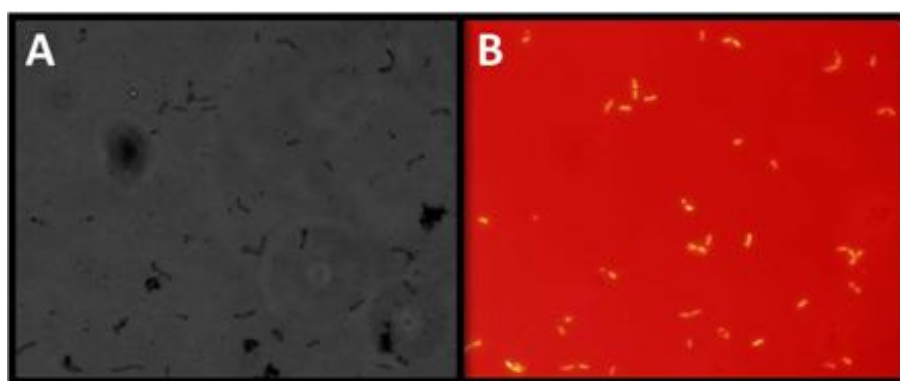
Fig. 3. (A) Phase contrast and (B) fluorescence microscopy of *Paracoccus* sp. LL1 cells grown using waste cooking oil for 96 h. Intracellular polyhydroxyalkanoate granules stained with Nile blue appears bright orange

3.3. Characterization of the poly (3HB-*co*-3HV) extract

The polymer extracted after batch culture fermentation was analyzed using FT-IR spectroscopy (Fig 4). IR spectra revealed the common characteristic features associated with PHA polymer. For instance, intense bands at 1280, 1727, and 3400-3600 cm^{-1}, are representing the features of -CH, ester carbonyl group, and hydroxyl group, respectively. Other bands at 1380, 1454, and 2931 cm^{-1} corresponding to -CH$_3$, -CH$_2$, and -CH groups, respectively, were also evident (Fig. 4). These results based on FT-IR data showed that the results are coherent and similar to previous reports [22].
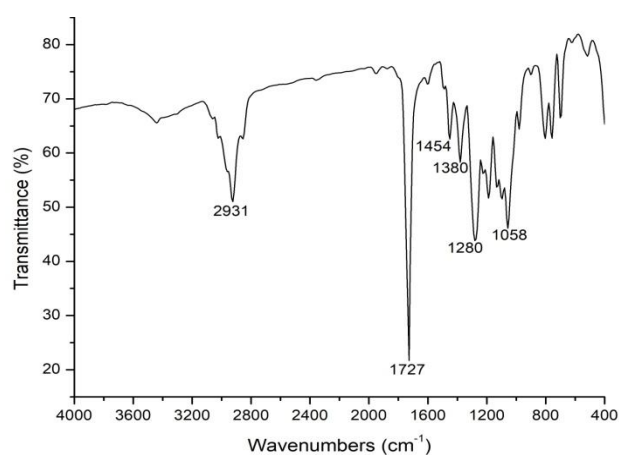
Fig. 4. FT-IR spectrum of polymer extracted from *Paracoccus* sp. LL1 grown using waste cooking oil for 96 h

4. Conclusion

The *Paracoccus* sp. strain LL1 used in the present work was capable of simultaneously producing carotenoids and PHA monomers using waste cooking oil. To the best of our knowledge, it is the first report that demonstrates bioconversion of waste cooking oil into astaxanthin-rich carotenoids and P (3HB-*co*-3HV). Here, it was also observed that use of surfactant to a minimal quantity can help in enhancing biomass and provide higher P (3HB-*co*-3HV) yield. It is evident that the produced PHA also contains a minor amount of 3HV within the polymer and its composition changes with culture conditions. The simultaneous production of these two bioproducts may subsidize the PHA production costs and will also be helpful to solve environmental problems related to oily waste discharge.

5. Acknowledgements

6. Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article

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جنس *پاراکوکوس* سوش LL 1 به عنوان کارخانه تک‌یاخته برای تبدیل ضایعات روغن پخت و پز به پلی‌هیدوروکسی‌آلکانوآت‌ها و کاروتنوییدها

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چکیده

سابقه و هدف: پلی‌هیدوروکسی‌آلکانوآت‌ها به عنوان جایگزین پلاستیک‌های بر پایه نفت توجه زیادی را به خـود معطوف داشته‌اند؛ اگرچه تولید صنعتی این ترکیبات با مواد خوراک دهی پرهزینـه بـه تـاخیر افتـاده اسـت. تولیـد همزمان سایر فرآورده‌های با ارزش به همراه پلی‌هیدوروکسی‌آلکانوآت توسط سوش‌های میکروبی می‌توانـد هزینـه کلی تولید را تا 50 درصد کاهش دهد. این مطالعه برای اولین بار نشان می‌دهد کـه پلـی‌هیدوروکسـی‌آلکانوآت و کاروتنوییدهای غنی از آستاگزانتین می‌توانند به طور همزمان توسط جنس *پاراکوکوس LL1* با اسـتفاده از ضـایعات روغن پخت و پز به عنوان زی‌مایه (substrate) تولید شود.

مواد و روش‌ها: گونه نمک دوست جنس پاراکوکوس *LL1* تحت شرایط ناپیوسته با استفاده از مکمل مواد معدنی محیط کشت با 1% (v v⁻¹) ضایعات روغن پخت و پز رشد داده شد. سـطح فعـال‌هـای (surfactants) گونـاگون بـه منظور بهبود مصرف زی مایه مورد استفاده قرار گرفتند. پلی‌هیدوروکسی‌آلکانوآت به دست آمده پس از هر تخمیر توسط میکروسکوپ فلورسنس، کروماتوگرافی گازی و طیف بینی مادون قرمـز تبـدیل فوریـه[1] مـورد بررسـی قـرار گرفت.

یافته‌ها و نتیجه گیری: استفاده از روغن به عنوان زی‌مایه به تولید 0/1 g l⁻¹ پلی(3-هیدوروکسی‌بوتیرات-کـو-3-هیدروکسی‌والرآت) با تولید همزمان 0/89 mg l⁻¹ کاروتنوییدها پس از 96 ساعت منجر شـد. هنگـامی کـه (v v⁻¹) % 0/1 توویین-80 به عنوان سطح فعال به منظور تسهیل سوخت و ساز روغن استفاده شـد، افـزایش 2/7 برابـری کـل ماده خشک سلولی به دست آمد. جنس پاراکوس *LL1* به عنوان کارخانه تک یاخته توانایی تبـدیل زیسـتی زی‌مایه‌های ارزان به فرآورده‌های با ارزش بالا را دارد.

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

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[1] Fourier transform infrared spectroscopy or FTIR