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Production of value-added Polyhydroxyalkanoates (PHA) by fungi using dairy wastewater as a carbon source

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Plastics are synthetic petrochemical-based polymers that take several decades to degrade due to their recalcitrant nature and often release numerous toxic byproducts on degradation. Alternatively, bioplastics (Polyhydroxyalkanoates (PHA)) are considered to be the best replacement for traditional plastics. They are biocompatible, environmentally friendly, biodegradable, and have physio-chemical properties similar to conventional plastics. The present study aimed to produce PHA using dairy wastewater as a substrate for microorganisms. Dairy waste is one of the common industrial wastes that needs to be properly disposed of to avoid water contamination. The PHA-producing ability of the organism was screened using Sudan Black B dye. The MSM medium amended with dairy wastewater (DWW) was used as a substrate for the organism for PHA synthesis. After incubation, the cell dry mass was measured, and PHA was extracted. The extracted PHA monomers were characterised by GC-MS analysis. The PHA-producing organism VITJK07 was identified as a *Candida tropicalis* species by 18S rRNA partial gene sequencing. The quantity of PHA synthesised by the organism was comparatively higher (21.4%) using dairy wastewater as a substrate than glucose (13.5%). The results of the study indicate that dairy wastewater could be used as a substrate for PHA production by the *Candida* species.

Keywords: Biopolymer, *Candida* species, Dairy wastewater, Plastics, Polyhydroxyalkanoates

IPC code; Int. cl. (2021.01)− C02F, C02F 3/30, CO2F 3/34, C02F 103/00

Introduction

Plastics are slow degraders (450 years for degradation of plastic bottles) due to their non-degradability, non-compatibility, and long durability[1]. It produces greenhouse gases, having a major negative impact on the environment globally[2]. Bioplastics are considered to be the best alternative to synthetic plastics. Polyhydroxyalkanoates PHA offers fascinating qualities for bioplastics manufacturing since they are thermoplastic, UV-resistant, elastic, gas barrier, stiff, hydrophobic, and biocompatible[3,4]. The combined forms of PHA, which include polyhydroxy butyrate-valerate (PHBV) and polyhydroxy butyrate (PHB), are non-toxic, durable, thermostable, and have the necessary strength[5]. PHA accumulates as granules in cell cytoplasm under extreme stress conditions like a smaller number of micro and macro elements like nitro phosphate, oxygen, magnesium, sulfate, and trace elements or in the presence of huge amounts of carbon source[6]. They are made up of hydroxyalkanoates and are classified into two categories based on the number of carbon atoms in the monomer units. They are medium chain length (mcl) Polyhydroxyalkanoates PHA consisting of 6-14 carbon atoms, like P(3HB-co-3HV) [poly-3-hydroxybutyrate-co-3-hydroxyvalerate] copolymer, whereas short chain length (scl) PHA consists of 3-5 carbon atoms, like P(3HB) [poly-3-hydroxybutyrate][7]. PHA breaks down completely and naturally to CO_2 and H_2O in the environment with the help of various microorganisms[8]. The synthesis of PHA by using various bacterial species is mostly determined by the type of carbon sources utilized[9]. Although different strains of bacteria have been found to use biowastes as a carbon source for PHA production, they are unable to grow effectively on waste carbon sources, which results in a decreased PHA yield[10]. PHA was also produced by yeast utilising biowaste as a carbon source. Ojha and Das[11] have reported that agricultural waste like teff straw, corn cob, sugarcane bagasse and industrial wastes like leather effluents, chicken feathers, and animal skins was used for PHA production. Apart from this fruit-vegetable wastes like orange, banana peels and waste fry oils like sesame, jatropha, palm oils, and dairy waste are considered as

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a biowaste material and also used for PHA production using microbes.

PHA can also be produced from fat- and sugar-rich substrates like agricultural, dairy, and household food waste that are present in water bodies as pollutants. These wastes can be utilised for PHA production to turn the waste into value-added products. PHA is extensively used in biomedical, industrial, and environmental applications like film and fabric production, soft tissue engineering, cartilage engineering, wound healing, cosmetics, packaging, as a drug carrier[12-14], etc. The aim of the current study was to use dairy waste as the substrate (carbon source) for PHA synthesis by the yeast and to analyse the PHA monomers produced.

Materials and Methods

Chemicals used for the experiment

Nutrient agar medium and chemical composition used for MSM medium were prepared as described by Jyothirmayee *et al.*[15]. All the culture media chemicals and Sudan black B dye (stain) powder were purchased from Hi-Media. All the solvents used for extraction were of HPLC grade and were purchased from Hi-Media, Mumbai, India.

Sample collection

Soil samples were collected from fertile agricultural land, Chandrappa Naidu Kandiga Village, Puttur Mandal, Tirupati District, Andhra Pradesh, India, for the isolation of potential PHA-producing organisms[15]. The Soil sample was collected using the sterilised spatula after removing the top layer in a labelled sterile bag, transported to the laboratory and stored at 4°C until further use. The raw wastewater of dairy was collected from the industry, Swarna Milk Dairy, Sivurajapalam, Puttur, Tirupati District Andra Pradesh, India. Samples were collected in sterilised bottles and then transported to the laboratory and stored at 4°C.

Isolation of potential isolates for PHA production

The enrichment process was used to isolate potential strains[16]. An agricultural soil sample (1.0 g) was taken and serially diluted up to 10^{-7}, and 1 mL of the sample was added into nutrient agar medium using the spread plate method followed by the streak plate method for the isolation of pure culture. The inoculated plates were incubated at 37°C for 48 hours. The pure culture obtained was stored at -20°C using glycerol stock[17].

Screening for PHA production

The Sudan black B staining method was used for screening the isolates for the presence of PHA/PHB granules[18,19]. The isolates were grown on a nutrient agar medium. The cultured plates were incubated at 37°C for 48 hours. Sudan black B staining solution was prepared by adding 0.02 g of Sudan black B dye in 100 mL of 70% ethanol[4]. After incubation, the staining solution was poured over the plates containing microbial colonies and kept uninterrupted for 30 minutes, and then the excess stain was washed with 96% ethanol[20]. The PHA producers appear in bluish-black colour colonies, and non-PHA producers appear in white colonies[21].

Phenotypic and Genotypic characterisation

The PHA-producing potential isolate was identified as having phenotypic and genotypic characterisation. The surface, texture, colour, elevation, and margin were examined under SEM (scanning electron microscope) (Carl Zeiss, model no: EVO/18, Vellore Institute of Technology, Vellore). The genotypic characterisation of the PHA-producing isolate was carried out by 18SrRNA partial gene sequencing. The extraction of DNA was carried out using a High pure PCR template preparation kit (Roche). The DNA was amplified using universal primers 18F 5'-TGTACACACCGCCCGTC-3' and 28R 5'-ATCGCCAGTTCTGCTTAC- 3' by using NCBI-BLAST (National Center for Biotechnology Informations – Basic local alignment search tool), the sequences acquired were aligned with known 18S rRNA genome sequences available in the GenBank database. MEGA 11 (Molecular evolutionary Genetic analysis) software[22] was used for the construction of a phylogenetic tree using the neighbour-joining method.

Extraction of PHA

The MSM medium was used for the cultivation of PHA-producing microorganisms. The composition of the medium in g/L is dipotassium hydrogen phosphate (K_2HPO_4): 2.27g/L, Potassium dihydrogen Phosphate (KH_2PO_4): 0.94g/L, Ammonium sulphate (NH_4SO_2): 0.67g/L, 1% glucose (carbon source)[15]. The loopful of culture was inoculated into a 500 mL conical flask containing 250 mL of MSM medium with DWW as a source of carbon and kept for incubation at 37°C, 200 rpm for 7 days. Glucose was also used as a carbon source for the isolate for PHA production without DWW, which served as a control.

After incubation, the PHA was extracted by centrifuging the culture medium at 7000 rpm (4°C) for 10 min. The pellet was collected and allowed to dry at 55°C in a hot air oven. The dry weight of biomass (m_1) was measured, and values were noted. After dissolving the pellet in 5% NaOCl solution, they were incubated for 1 hour at 37°C. After adding chloroform (50 mL), the mixture was kept at 37°C for an additional one hour. The mixture was centrifuged, and the top layer was discarded. The PHA containing the lower phase was diluted in chloroform and ice-cold methanol in the ratio 9:1 (V/V) to facilitate the precipitation of PHA in the solution. The PHA precipitate-containing solution was vortexed for 2 min and centrifuged at 10000 rpm for 10 min; the PHA pellet obtained was dried at 40°C. The purified PHA weight (m_2) was calculated and analysed by Gas chromatography-mass spectrometry (AGILENT; GC-7890A & MS-5975C). The following formula was used to determine the PHA content.

$$PHA\% = {}^{m2}/_{m1} \times 100$$

Results

Sample collection and isolation of PHA/PHB-producing isolates

A total of twelve isolates, including three bacterial, six actinomycetes, two yeast, and one fungus, were obtained from the soil samples. All the isolates were screened for PHA production. The PHA-producing isolate VITJK07 appeared bluish-black in colour after Sudan Black B staining, whereas the non-PHA producers appeared white in colour (Fig. 1).

Phenotypic characterisation of PHA-producing isolate

The PHA-producing isolate was morphologically characterised. It is round in form, has a smooth surface, moist in texture, opaque in colour, has raised elevation, and has an entire margin. The isolate showed the bud formation characteristic of yeast under SEM with 16.61KX magnification (Fig. 2).

Molecular characterisation of PHA-producing isolate

The 18S rRNA partial gene sequence of the isolate VITJK07 (GenBank accession number OQ376565.1) was aligned with the sequences available in the GenBank database. A BLAST search result showed that the strain belonged to the genus *Candida*. It showed a sequence similarity of 99.34% with *Candida tropicalis* strain MYA-3404 (CP047875.1). Hence, the PHA producing isolate (VITJK07) was designated as *C. tropicalis* sp. VITJK07.

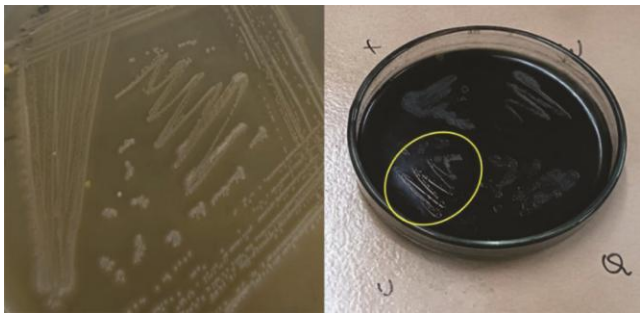

Fig. 1 — PHA-producing isolate VITJK07 showed a bluish-black colour after staining with Sudan black B dye.

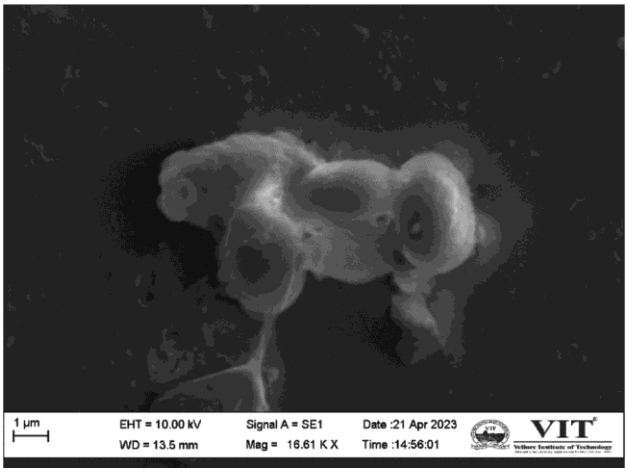

Fig. 2 — PHA-producing isolate VITJK07 showed bud formation under SEM analysis.

Phylogenetic analysis

The phylogenetic tree was constructed by using the neighbor-joining method. It showed the relationship between PHA-producing isolate VITJK07 and the closest genera *C. tropicalis* strain MYA-3404 (Fig. 3).

PHA production and extraction

The isolate VITJK07 was cultivated in 500 mL Erlenmeyer conical flasks, each containing 250 mL of MSM broth with DWW as a carbon source, and incubated at 37°C for 7 days in an orbital shaker at 200 rpm. Glucose (1%) was used as a substrate (control) without dairy wastewater. After incubation, the cultures were centrifuged to obtain the pellet and dried. When glucose and DWW were used as a substrate, 3.1 and 2.1 g/L of cell dry biomass (CDW) were obtained, respectively. The yield of PHA was calculated as 13.5 and 21.4% for glucose and DWW, respectively (Fig. 4).

GC-MS analysis of PHA-produced by VITJK07

The PHA produced by *C. tropicalis* sp. VITJK07 was analysed by Gas chromatography-mass

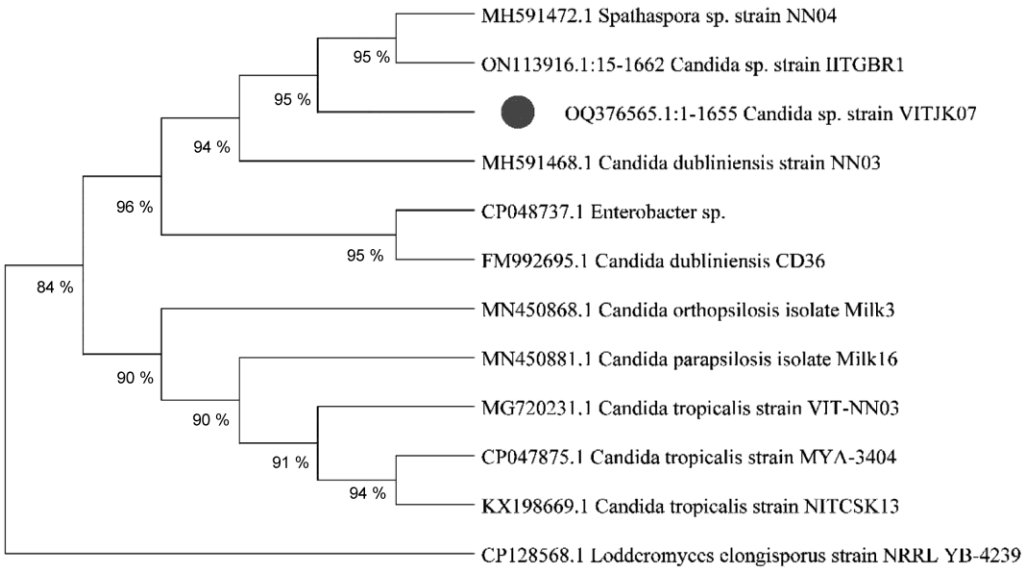

Fig. 3 — The phylogenetic tree of the PHA-producing isolate VITJK07 (OQ376565.1) was constructed using MEGA software.

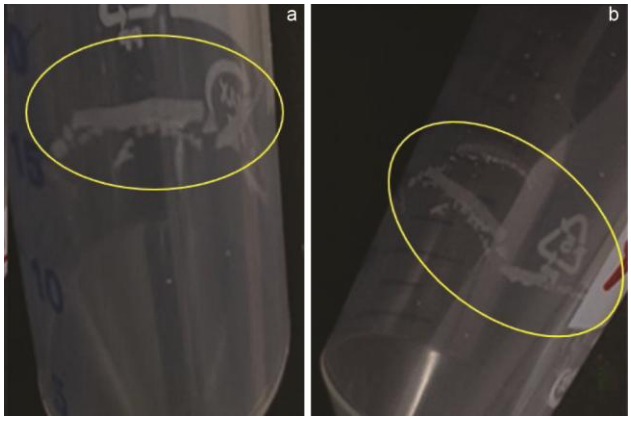

Fig. 4 — PHA produced by the isolate VITJK07 using different substrates. a) Glucose, and b) Dairy wastewater.

spectroscopy to determine the presence of PHA monomeric compounds. When glucose was used as a carbon source, few characteristic peaks (Fig. 5a) were detected with retention times of 7.94, 18.13, 18.67, 19.99, and 20.54 min corresponding to butanedioic acid, dimethyl ester (0.94%), hexadecanoic acid, methyl ester (1.60%), n-hexadecanoic acid (5.40%), 11-Octadecenoic acid, methyl ester, (Z)- (1.74%), octadec-9-enoic acid (3.33%), hexadecanoic acid, 2-(acetyloxy)-1,[(acetyloxy)methyl] ethyl ester (1.36%) (Table 1). When dairy wastewater was used as a carbon source, few characteristic peaks (Fig. 5b) were detected with a retention time of 16.34, 18.41, 20.09, 20.30, 25.24 min corresponding to tetradecanoic acid (1%), n-Hexadecanoic acid (6.02%), 9-Octadecanoic caid (5.95%), Octadecanoic acid (2.50%) (Table 1).

Discussion

To promote a circular bio-economy and to combat plastic pollution, waste from different sources can be valorised to produce commercially important products. PHAs are biobased polyesters with mechanical properties similar to conventional plastics. Therefore, they can be replaced with recalcitrant petroleum-based plastics. In general, they are accumulated as granular lipids in the microbes. Currently, PHA production is considered to be expensive and can be overcome by utilising low-cost substrates. Dairy wastes are one of the low-cost yet nutrient-rich substrates for microbial growth. There are reports on the production of PHA using dairy waste by microorganisms such as *Pseudomonas hydrogenovora*, *Hydrogenophaga pseudoflava*, and *Haloferax mediterranei*[23]. Production of PHAs, P(3HB) homopolymer AND P(3HB-co-3HV) copolymer by *Halomonas alkaliantarctica* using a dairy waste (cheese whey and cheese whey mother liquor) was reported recently[24]. Production of PHA and other byproducts, including exopolysaccharides, pigments, enzymes, bio-surfactants, and biofuels, was also reported recently[25]. PHA was also produced by *Pseudomonas aeruginosa* and *Bacillus subtilis* using cheap carbon sources, molasses and banana peels[26]. PHA was also produced by using agro wastes (corncob, plantain peduncle and sugarcane bagasse) as carbon sources[27]. It was reported recently that cacao shells, cheese whey, wine, wood, and beet molasses were used for PHA production[28]. Organically enriched soil is found to be one of the

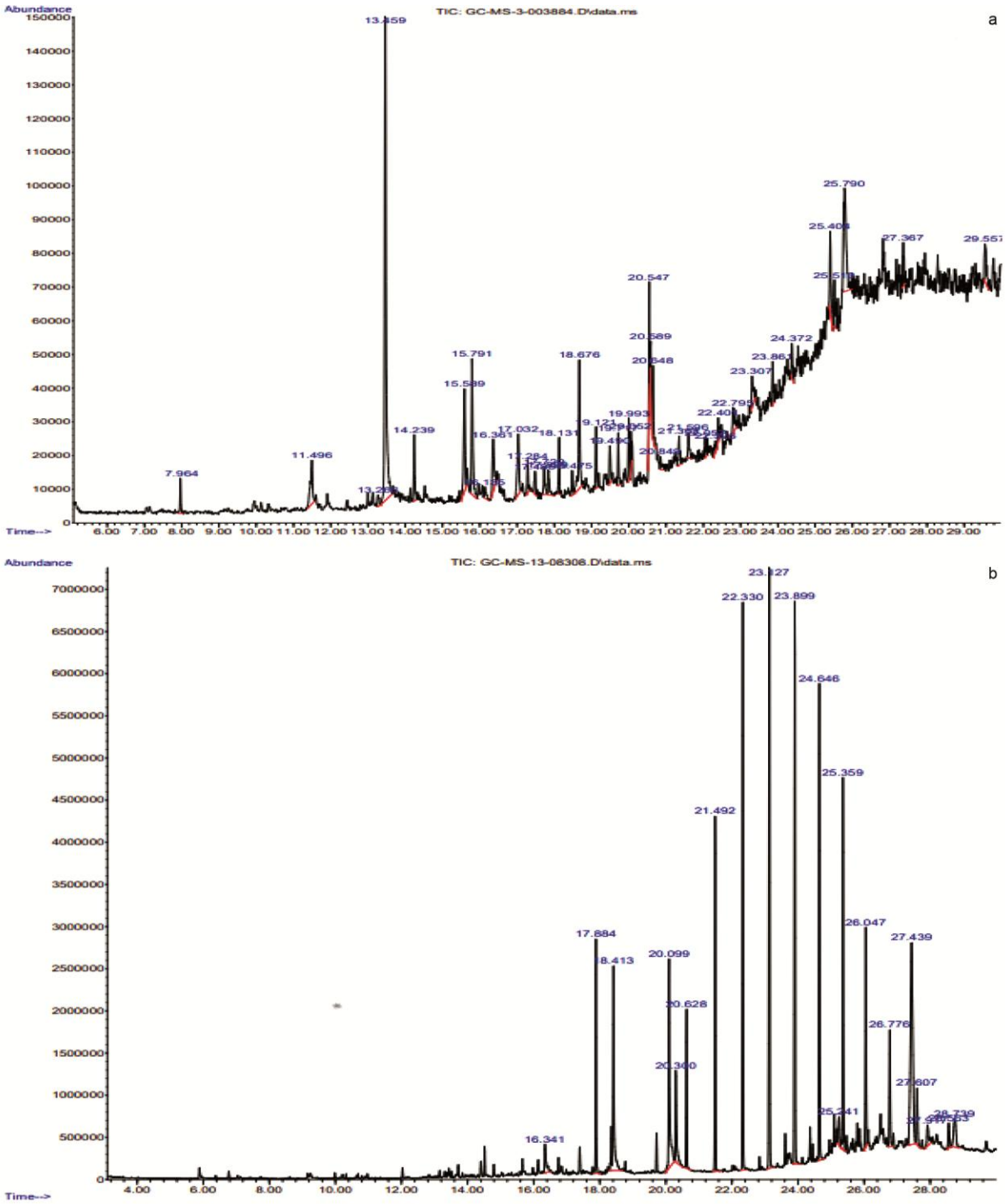

Fig. 5 — GC-MS spectra of PHA produced by VITJK07. a) Glucose, and b) Dairy wastewater as a carbon source.

rich sources of PHA-producing microorganisms. In the present study, VITJK07 was identified as the potential producer of PHA by the Sudan black dye screening method. Morphological and molecular taxonomic characterisation revealed that the isolate VITJK07 was a yeast species and showed maximum similarity (99.34% similarity) to *C. tropicalis* strain MYA-3404. Moreover, *C. tropicalis* was reported to be more prevalent in soil samples[29]. Evidently, several yeast species like *Candida norvegensis*[30], *Candida*

Table 1 — GCMS analysis of PHA monomers produced by VITJK07 using different substrates

Compound	Retention time (RT)	Area of percentage (%)	Molecular weight (g/mol)	Molecular Formula
Glucose				
Butanedioic acid, dimethyl ester	7.964	0.94	146.14	$C_6H_{10}O_4$
Hexadecanoic acid, methyl ester	18.131	1.60	270.5	$C_{17}H_{34}O_2$
n-Hexadecanoic acid	18.676	5.40	256.42	$C_{16}H_{32}O_2$
11-Octadecenoic acid, methyl ester, (Z)-	19.993	1.74	296.5	$C_{19}H_{36}O_2$
Octadec-9-enoic acid	20.547	3.33	282.5	$C_{18}H_{34}O_2$
hexadecanoic acid, 2-(acetyloxy)-1, [(acetyloxy)methyl] ethyl ester	23. 861	1.36	414.6	$C_{23}H_{42}O_6$
Dairy wastewater				
n-Hexadecanoic acid	18.413	6.02	256.42	$C_{16}H_{32}O_2$
9-Octadecanoic acid	20.099	5.95	282.5	$C_{18}H_{34}O_2$
Octadecanoic acid	20.300	2.50	284.5	$C_{18}H_{36}O_2$

bombicola[31], *C. tropicalis* strain KY72 and *Pichia kudriavzevii* TSLS243[(Ref. 32)] have been reported to be PHA producers.

Microorganisms produce PHA monomers and precursors, which can be polymerised further to make bioplastics. PHA produced by VITJK07 on glucose and dairy waste was extracted and characterised by GC-MS. Higher amounts of PHA monomers, such as hexadecanoic acid and octadecanoic acid, were produced by VITJK07 when dairy waste was used as a carbon source. This may be due to the adoption of different metabolic pathways for PHA production when different carbon sources are used as a substrate. The yield of PHA from dairy waste and glucose was 21.4 and 13.5% g/L, respectively.

Industries are manufacturing low-cost green plastic by using/recycling the waste as a cheap carbon source for PHA production[33]. The circular economy has the potential to improve process performance, resource recovery efficiency, market price reduction, and waste stabilization[34]. Domingos *et al*.[35], showed that integrated whey valorisation can also make a platform for circular bioeconomy and reduce commercialisation risk. In their study, the cheese whey was first fermented an aerobically, yielding 12.55 g/L of volatile fatty acid (VFA). The VFA concentration was then thickened to 63 g/L using an electrodialysis technique. *Cupriavidus necator* was used to thicken VFA as a substrate to create PHA at a rate of 0.60 gPHA/gram of VFA. Colombo *et al.*[36] described an integrated biorefinery utilising deproteinised cheese whey wastes to generate PHA and biohydrogen. On the other hand, PHA-containing microbial cells can be offered to animals as feed, boosting their immune systems and later extracting PHAs from their faeces. The processed byproducts can be utilised to manufacture high-value products, including biostimulants, flocculants, animal feed, adhesives, protein hydrolysates, and other pharmaceutical products. PHAs are also employed in the production of amino acids, proteins, lipids, minerals, vitamins, and pigments for a variety of biological applications[15].

PHA's biodegradable and biocompatible characteristics make it an extremely sought-after product in the agricultural and biomedical fields. The high cost involved in PHA production is associated with the type of carbon source used[37]. The restricted market share of PHA companies, despite significant demand in terms of environmental concerns, has forced scientists to look for more research avenues to produce PHA economically. As a result, new production techniques have been developed, such as using diverse feedstock such as organic wastes and altering the PHA synthesising enzyme. Modifying the metabolic pathways for PHA synthesis with new tools such as CRISPR/Cas9 technology has simplified the procedure of synthesising PHA. Green solvents under pressure, supercritical solvents, hypotonic cell disintegration, and ionic liquids were used for the release of PHA granules. Apart from this switchable anionic surfactants and digestion of non-PHA biomass by animals are a few novel PHA recovery strategies that play a major role in the sustainable production of PHA[38]. However, knowledge of bioprocess and bioseparation technology may improve the quantity and quality of PHA production. A genome-scale metabolic model for predictive metabolic modelling and dynamic flux balance analysis has been suggested for large-scale bioplastic production[39].

Conclusion

In this study, the organisms VITJK07 isolated from the agricultural soil sample were found to be a potential producer of PHA using DWW. The production of PHA monomers by the isolate was confirmed by GC-MS analysis. The major PHA monomers obtained were tetradecanoic acid (1%), n-hexadecanoic acid (6.02%), 9-octadecanoic acid (5.95%), and Octadecanoic acid (2.50%) when DWW was used as a substrate. The results of this study suggest that dairy waste can be used as a cheap carbon source for the production of PHA using *C. tropicalis* sp. VITJK07. These monomers can be explored further for commercial use.

Conflict of interest

The authors declared no conflict of interest.

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