

Article

Production of biodegradable polymer from agro-wastes in *Alcaligenes* sp. and *Pseudomonas* sp.

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Abstract: The present study was aimed to evaluate the suitability of agro-wastes and crude vegetable oils for the cost effective production of poly- β -hydroxybutyrate (PHB), to evaluate growth kinetics and PHB production in *Alcaligenes faecalis* RZS4 and *Pseudomonas* sp. RZS1 with these carbon substrates and to study the biodegradation of PHB accumulated by these cultures. *Alcaligenes faecalis* RZS4 and *Pseudomonas* sp. RZS1 accumulate higher amounts of PHB corn (79.90% of dry cell mass) and rice straw (66.22% of dry cell mass) medium respectively. The kinetic model suggests that the *Pseudomonas* sp. RZS1 follows the Monod model more closely than *A. faecalis* RZS4. Both the cultures degrade their own PHB extract under the influence of PHB depolymerase. Corn waste and rice straw appear as the best and cost-effective substrates for the sustainable production of PHB from *Alcaligenes faecalis* RZS4 and *Pseudomonas* sp. RZS1. The biopolymer accumulated by these organisms is biodegradable in nature. The agro-wastes and crude vegetable oils are good and low cost sources of nutrients for the growth and production of PHN and other metabolites. Their use would lower the production cost of PHN and the low cost production will reduce the selling price of PHB based products. This would promote the large scale commercialization and popularization of PHB as ecofriendly bioplastic/biopolymer.

Keywords: Biodegradable plastic; Biodegradation; Characterization; Extraction; Kinetics; Production.

1. Introduction

Bountiful use of non-degradable synthetic polymers has created a frightening scenario for the environment. Synthetic polymers possess numerous health and environmental hazardous at every stage of their existence. The environmental and health concerns brought poly- β -hydroxybutyrate (PHB) as sustainable and the best alternative to the synthetic plastic and an ideal material for making biodegradable plastics. Moreover, it is completely biodegradable in a natural environment after disposal [1,2]. Although PHB has been found as an eco-friendly biopolymer, the cost associated with carbon substrate (50% of the overall production) used in the fermentation, has been a major limiting factor in the commercialization of biodegradable polyesters [3,4].

PHB production from various oils like oil from spent coffee grounds by *Cupriavidus necator* H16 [5], soybean oil by *Ralstonia eutropha* [6], olive oil, corn oil by *Pseudomonas* sp. [7] and palm oil by *R. eutropha* [8,9] has been used for PHB production. Similarly, PHB production from low cost and renewable carbon sources like industrial wastes such as malt, soya, sesame, molasses, bagasse, coconut pulp, and pharmaceutical waste have been reviewed and reported [10]. However, such substrates have resulted in less PHB yield and hence PHB production from inexpensive agricultural sources with high productivity may contribute significantly to lowering the production cost of PHB [11].

During nutrient starvation, organisms that accumulate PHB also degrade to get carbon and energy [1]. Such organisms having the potential of accumulating large amounts of PHB under carbon excess and nitrogen-deficient conditions and 90 capable of degrading this reserve food are of great commercial potential both in the production and degradation of biopolymer after its disposal [2]. The present study was aimed to evaluate agro-wastes and vegetable oils as potential substrates for higher yields of PHB by using *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1. Since these strains were earlier reported to produce copious amounts of PHB in nitrogen deficient medium (NDM) [12,13], they were used to check PHB production from agro-wastes and vegetable oils. The present research was carried out in Microbiology Department of PSGVP Mandal's College, Shahada, India during July 2018 to January 2020.

2. Results

2.1. Screening and detection of PHB production

Growth of *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 appeared in the form of black blue colored colonies. Bright field microscopic observation of Sudan black B stained smears revealed the presence of bright refractile granules.

2.2. Growth and PHB accumulation as a function of time

A. faecalis RZS4 exhibited a lag phase of 6 h, exponential phase from 6 to 24 h, static phase from 24 h to 30 h followed by decline phase. PHB accumulating began from 6 h and continued up to 48 h. However, an optimum amount of PHB accumulation occurred at 24 h (the late log phase) (56.92% of dry cell mass i.e. 5.2 g/L) (**Figure1a**). *Pseudomonas* sp. RZS1 exhibited a lag phase of 6 h, exponential phase from 6 h to 30 h, static phase from 30 h to 36 h followed by decline phase. PHB accumulation began from 12 h and continued up to 48 h. Maximum PHB accumulation occurred during the late log phase 30 h) (54.14% of dry cell mass, 4.2 g/L) (**Figure1b**).

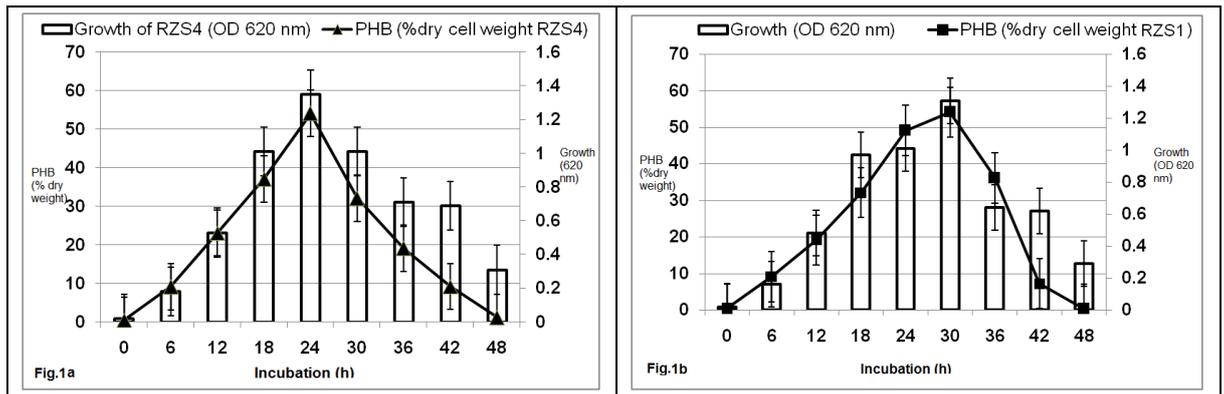


Figure 1. Growth and PHB accumulation in a) *A. faecalis* RZS4 b) during growth in NDM broth at 30 °C for 48 h at 120 rpm. Samples were withdrawn at 6 h intervals and were subjected to the estimation of cell growth and PHB.

2.3. Kinetics of biomass growth and PHB formation

The differential equations were solved using the 'odeint' solver of the 'scipy' module of the Python 3.7 with the internal conditions of 0.1, 30, and 0 for the biomass, substrate and product concentration respectively. The solution of the differential equation was fitted with the experimental data to calculate the kinetic parameters. The mathematical model fitted the experimental data of biomass growth and PHB production of both the micro-organism with good accuracy. The kinetic model of the *Pseudomonas* sp. showed a very good fit for the biomass growth ($R^2 = 0.94$, RMSE = 0.049) and PHB production ($R^2 = 0.99$, RMSE = 0.135). The model predicted kinetic parameters of the *Pseudomonas* sp. are $\mu_{max} = 2.03 \times 10^{-1}/h$, $K_s = 30$ g/L, $\alpha = 8.63$, $\beta = 3.11 \times 10^{-14}$, $Y_{x/s} = 9.56 \times 10^{-2}$ g biomass/g substrate, $Y_{p/s} = 1.74 \times 10^0$ g PHB/g substrate (Figure 2a). The *A. faecalis* RZS4 data also showed a good fit with the model for both biomass production ($R^2 = 0.78$, RMSE = 0.079) as well as the PHB formation ($R^2 = 0.98$, RMSE = 0.24) (Figure 2b). The kinetic parameters for the *A. faecalis* RZS4 predicted by the model are $\mu_{max} = 8.0 \times 10^{-2} h$, $K_s = 3.08$ g/L, $\alpha = 14.99$, $\beta = 1.88 \times 10^{-10}$, $Y_{x/s} = 8.64 \times 10^{-1}$ g biomass/g substrate, $Y_{p/s} = 1.69 \times 10^{-1}$ g PHB/g substrate. The kinetic model assumed that both the organisms follow Monod model for the growth.

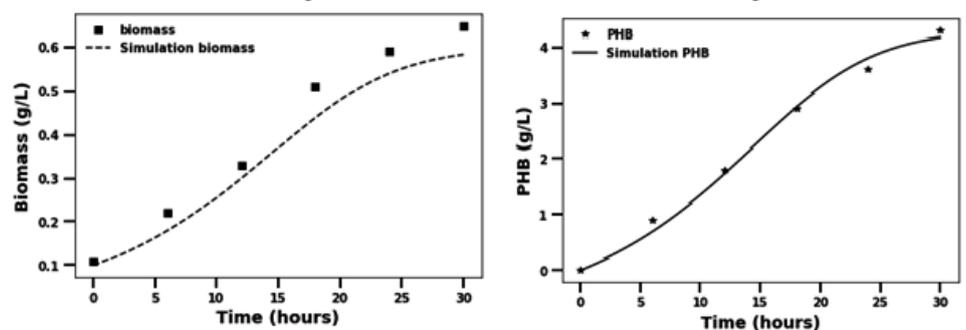


Figure 2a. Kinetics of biomass growth and PHB formation by *Pseudomonas* sp. RZS4

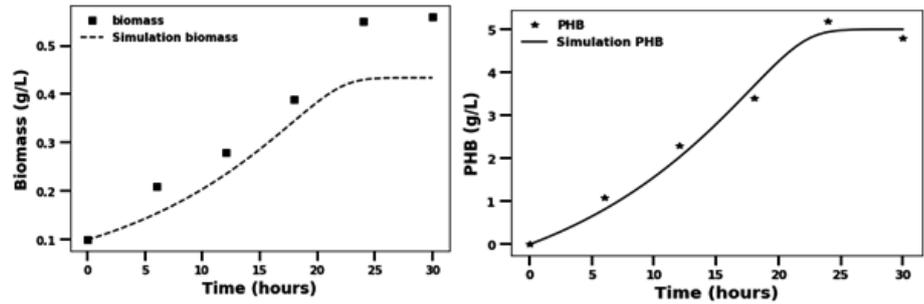


Figure 2b. Kinetics of biomass growth and PHB formation by *A. faecalis* RZS1

2.4. Extraction and estimation of PHB and cell mass

For intracellular PHB, cell mass was incubated in sodium hypochlorite (1% v/v) at 37 °C for 1 h and extracted with a mixture of acetone: ethanol (1:1). White crystalline powder of PHB leftover after evaporation of chloroform confirmed the extraction of PHB. The amount of PHB of *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 was gravimetrically estimated as 79.90% of dry cell mass 66.22% of dry cell mass respectively. Spectrophotometric estimation of PHB produced by these organisms was 5.21 g/L and 4.32 g/L respectively.

2.5. Production of PHB from agro-waste

For sustainable production of PHB, various agro-wastes were checked for their suitability to support the production of PHB from these renewable and cheap carbon sources. *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 were separately grown at 30 °C for 48 h at 120 rpm in each NDM amended with 20 g/L of corn waste, rice straw, and wheat straw revealed that *A. faecalis* RZS4 yielded more PHB in medium containing corn waste as carbon source (5.21 g/L, i.e. 541.46 µg of PHB/mg of cell mass). Whereas *Pseudomonas* sp. RZS1 produced maximum PHB in medium amended with rice straw as carbon source (4.31 g/L i.e. 379.98 µg of PHB per mg of cell mass). *A. faecalis* RZS4 yielded more PHB but less growth with these agricultural wastes while *Pseudomonas* sp. RZS1 grew luxuriously but produced less PHB.

2.6. Production of PHB from crude vegetable oils

A. faecalis grown at 30 °C for 48 h at 120 rpm in NDM individually amended with 20 g/L of vegetable oil, yielded a higher level of PHB in NDM containing sesame oil (5.92 g/L, 66.21% of dry cell mass, 368.98 µg of PHB/mg of cell mass) (**Figure 3a**). *Pseudomonas* sp. RZS1 also produced higher PHB yield in NDM amended with sesame oil as carbon source (5.48 g/L, i.e. 69.52% of dry cell mass equivalent to 333.33 µg of PHB/mg of cell mass) (**Figure 3b**).

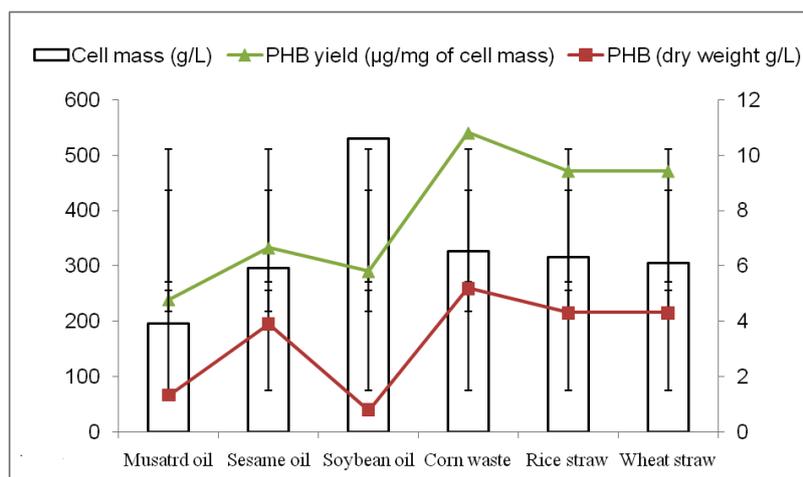


Figure 3a. PHB accumulation in *A. faecalis* RZS4 during 24 h growth at 30 °C and 120 rpm in NDM separately amended with crude vegetable oil and agro-wastes (@20 g/L) as carbon substrates.

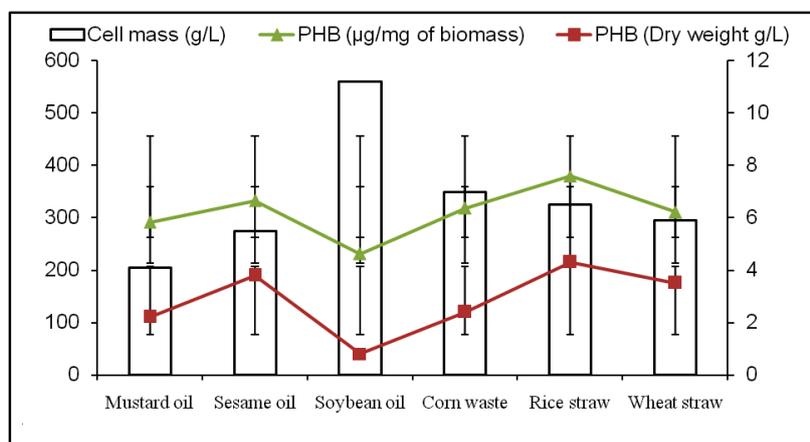


Figure 3b. PHB accumulation in *Pseudomonas* sp. RZS1 RZS4 during 30 h growth at 30 °C and 120 rpm in NDM separately amended with crude vegetable oil and agro-wastes (@20 g/L) as carbon substrates.

2.7. Characterization of PHB

2.7.1 UV-Visible spectrophotometry

A. faecalis RZS4 produced more amounts of PHB than *Pseudomonas* sp. RZS1 and hence its PHB extract was subjected to UV-Visible spectrophotometric characterization. UV-Visible spectrophotometric studies of PHB extract of *A. faecalis* RZS4 revealed the presence of a single peak at 220 nm with an absorbance value of 3.436 (**Figure 4**).

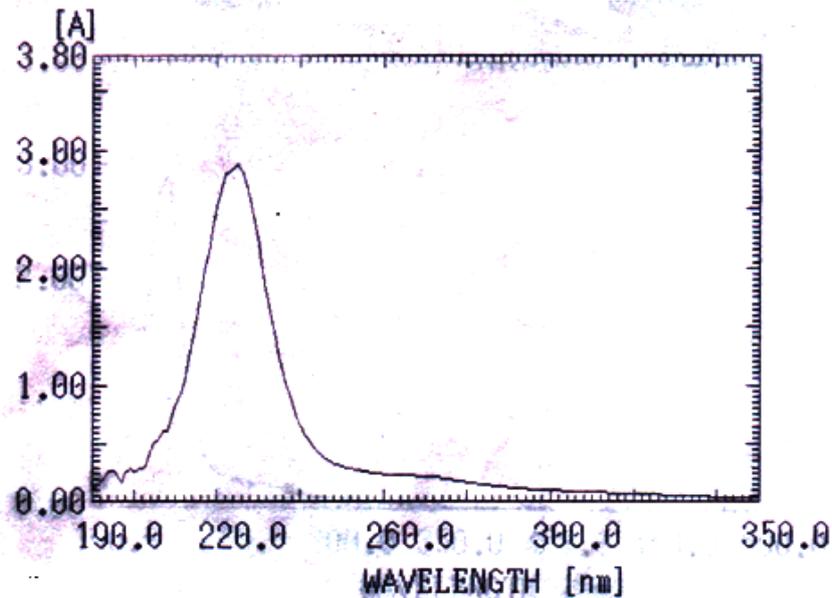


Figure 4. UV-Vis spectra of PHB extract of *A. faecalis* RZS4 obtained from NDM and extracted with acetone : alcohol.

3.8. Assessment of biodegradation of PHB

The growth of *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 in MM amended with PHB extract as the only carbon source resulted in the formation of zone of PHB hydrolysis (**Figure 5a**). *A. faecalis* RZS4 produced a bigger zone of PHB hydrolysis vis-à-vis *Pseudomonas* sp. RZS1 (**Figure 5b**). While no zone of hydrolysis was observed in MM lacking PHB (**Figure 5b**).

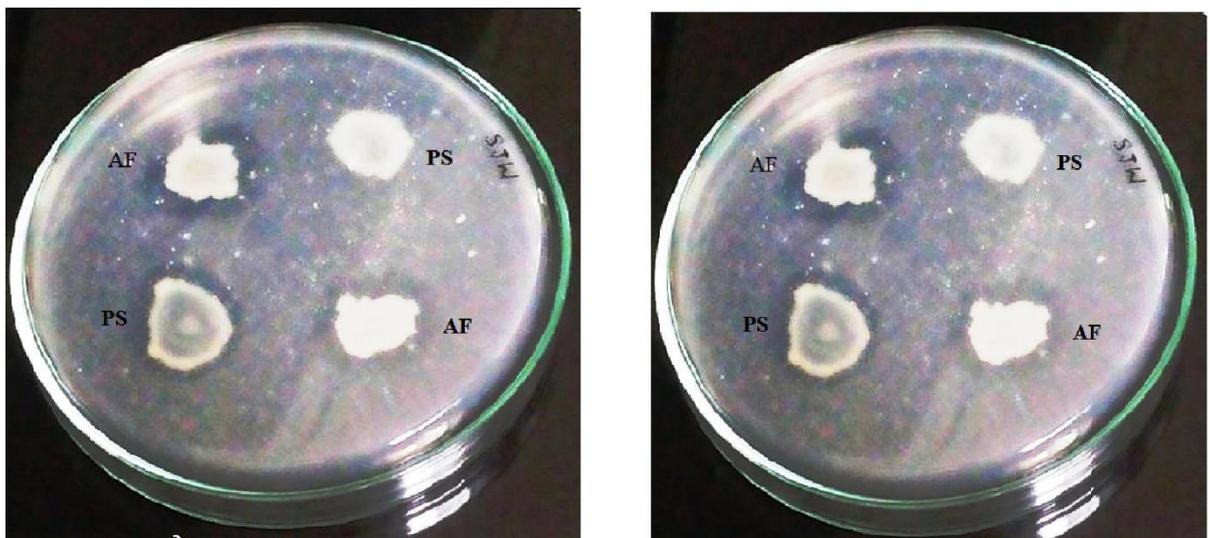


Figure 5. Biodegradation of PHB extracts of *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1. *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 were grown on MM having PHB extract (as the sole source of carbon grown) for 48 h at 30°C. After the incubation, a clear zone of PHB hydrolysis around the colony was observed (**Figure 5a**) while no zone of hydrolysis was observed in control i.e. MM without PHB (**Figure 5b**).

PHB added in NDM medium and inoculated with *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 exhibited visible changes in the surface morphology i.e. roughening of surface and

formation of holes and distortion of PHB film (**Figure 6a**) as against no changes in the surface morphology of the film (**Figure 6b**).



Figure 6. Roughening of the surface morphology and formation of holes in PHB film due to the action of *Pseudomonas* sp. RZS1 and *A. faecalis* RZS4 in NDM indicated the biodegradation of PHB film Figure 6a). Control preparation (NDM +PHB film) did not show any change in the morphology of the film (**Figure 6b**).

2.9. Assay of PHB depolymerase enzyme

PHB depolymerase activity in the case of *A. faecalis* RZS1 was higher (7.690 U) as compared to *Pseudomonas* sp. RZS1 (6.511 U).

3. Discussion

3.1. Screening and Detection of PHB production

The intensity of black-blue color of the colonies on MM reflects the degree of PHB accumulation and has been reported as one of the best and rapid screening methods to detect the presence of PHB granules in the cytoplasm of PHB accumulating bacteria [14-16]. The appearance of bright refractile granules in Sudan black B stained smears under a bright-field microscope has been reported as a confirmatory method of screening PHB positive organisms [17].

3.2. Growth and PHB accumulation as a function of time

Maximum growth and PHB accumulation by *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 in CRGM is attributed to the rich availability of carbon in this medium and deficiency of nitrogen in NDM. While a decline in PHB after 24 and 30 h incubation in *A. faecalis* RZS1 and *Pseudomonas* sp. RZS1, respectively is due to the exhaustion of available nutrients that causes the organism to mobilize the PHB storage [17]. Bacteria capable of accumulating PHB have been reported to mobilize intracellular PHA [18]. Accumulation of optimum amount of PHB during the late log phase has been reported in *A. faecalis* and *Microbacterium* sp. [17,12].

3.3 Kinetics of biomass growth and PHB formation

The experimental data of the *Pseudomonas* RZS1 showed a very good fit with the model as compared to the *A. faecalis* RZS4. This suggests that the *Pseudomonas* sp. species follows the Monod model more closely as compared to *A. faecalis*

3.4. Extraction and estimation of PHB and cell mass

Hypochlorite digestion of non-PHB cell mass followed by extraction with a 1:1 mixture of ethanol and acetone has been reported as the best and method for the extraction of PHB. Hypochlorite digests non-PHB cell mass without affecting PHB [12]. They claimed ethanol and acetone mixture as high yielding methods as compared to other extraction and recovery methods.

3.5. Production of PHB from agro-waste

Production of PHB in NDMM amended with corn waste, rice straw, and wheat straw by both the cultures indicated the potential of the organisms to utilize these substrates as carbon sources. Gowda and Shivakumar [19] reported the production of poly hydroxyl alkanates (PHA) by *Bacillus thuringiensis* IAM 12077 grown in various agro-wastes such as rice husk, wheat bran, bagasse, and straw as low-cost carbon substrates. Several studies recorded PHB accumulation in a wide variety of bacteria grown on agro-wastes [20,21]. Agro-wastes have been suggested as cheaper and renewable carbon substrates for the economical production of PHB. These wastes are generated abundantly and serve as rich sources of carbohydrates. Kulkarni et al. [22] reported the maximum accumulation of PHB in *Halomonas campisalis* MCM B-1027 with bagasse. Getachew and Woldesenbet [23] found optimum PHB accumulation in *Bacillus* sp. grown on cane bagasse, corn cob, and banana peel. Alsheherei [24] reported the production of PHB from fruit peel by using different species of *Bacillus* and found a good yield of PHB (47.61%).

3.6. Production of PHB from crude vegetable oils

The synthesis of PHB using vegetable oils offers the possibility of generating a wide variety of free fatty acids and thus the chances of producing a wide variety of PHB having different copolymer constituents that may have great commercial potential.

Vegetable oils are known to provide essential moieties of fatty acids that are required as precursors in the biosynthesis of PHA. There are reports on the production of PHB from olive oil, corn oil [7], palm oil [9]. Arun et al., [25] reported PHB production in sesame oil by *Alcaligenes eutrophus*. Song et al. [26] reported production of the optimum amount of intracellular PHA in *Pseudomonas* sp. strain DR2 grown in NDM containing vegetable oil as the sole carbon source. Although various oils have been used for PHB production, however, the PHB yield has been limited to 37-40% [27]. Thakor et al. [28] reported the accumulation of medium chain length PHA in a medium that contained olive oil, and sesame oil. More growth of *Pseudomonas* sp. in vegetable oils may be due to its ability to degrade oils and fats.

The suboptimal yield of PHB from agro-waste by both isolates reflected their inability to produce copious amounts of PHB vis-à-vis PHB yield obtained from vegetable oils. More PHB productivity from vegetable oils maybe because of the fatty acids contents of the oils. Fatty acids such as erucic acid, oleic acid, palmitic acid, palmitoleic acid, stearic acid, and linoleic acid found in mustard and sesame oil are the precursors in the biosynthetic pathway of PHB. Higher PHB yield by *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 from sesame and mustard oil indicated the diverse metabolic potential of these isolates.

3.7. Characterization of PHB

3.7.1 UV-Visible spectrophotometry

The presence of single peak at 220 nm with an absorbance value of 3.436 corresponds to

the peak and absorbance of PHB [30]. PHB and copolymers are also known to absorb between 230-254 nm [25]. The presence of characteristic absorption maxima indicated the presence of a single type of PHA.

3.8. Assessment of biodegradation of PHB

To be an eco-friendly biopolymer, the PHB produced by the isolates should be biodegradable. The appearance of zone of PHB hydrolysis in MM medium containing PHB as the only source of carbon indicated the ability of these cultures to degrade their own PHB extract. No zone of hydrolysis on MM medium without PHB is due to the absence of growth due to lack of carbon source (PHB). More hydrolysis of PHB by *A. faecalis* RZS4 indicated more biodegradation potential of PHB as compared to *Pseudomonas* sp. RZS1. This may be due to the more PHB depolymerase activity (7.690 U) in *A. faecalis* RZS4 as compared to *Pseudomonas* sp. RZS1 (6.511 U). Sayyed and Chincholkar [17] reported the production of higher amounts of PHB in *A. faecalis* and it is known that the PHB depolymerase activity is proportional to the amount of PHB accumulated by the cell. Therefore the higher PHB depolymerase activity is because of the accumulation of more amounts of intracellular PHB by *A. faecalis* RZS1.

Roughening of surface, formation of holes and distortion of PHB film in NDM medium separately inoculated by *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 indicate the biodegradation of polymer while no changes in the surface morphology of the film PHB film in uninoculated medium (NDM+PHB film) rule out the possibility of autolysis or auto-oxidation of PHB.

The ability of organisms to hydrolyze PHB reflects their potential to produce PHB depolymerase. Mergaert et al. [30] observed PHB degradation by the clear zone method in 295 different soil isolates. Elbanna et al. [32] reported a similar observation on PHB degradation in *Schlegelella thermodepolymerans* and *Pseudomonas indica* K2. Sayyed and Gangurde [33] reported PHB accumulation in *Pseudomonas* sp under aerobic and anaerobic conditions. Sayyed et al., [12,13] reported biodegradation of PHB by a *Stenotrophomonas* sp. and *Microbacterium* sp.

3.9. Assay of PHB depolymerase enzyme

The enzyme assay is based on the fact that PHB extract exposed to PHB depolymerase (supernatant) under the controlled buffered system, causes degradation of PHB polymer into monomers. Sayyed et al. [12] reported 6.675 U/mg/mL PHB depolymerase in *Microbacterium paraoxydans* RZS6. After 48 h (log phase) of incubation, RZS6 produced 6.675 U of PHB depolymerase with 0.247 mg/mL protein content in 2 days at 30 °C. Gowda and Srividya [19] reported the production of 4 U of extracellular PHB depolymerase with a protein content of 0.05 mg/mL from *Penicillium expansum*. After 4 days of incubation at 30 °C at 120 rpm in MSM, *Stenotrophomonas* sp. RZS7 yielded 0.721 U/mL PHB depolymerase. The yield of PHB depolymerase in *A. faecalis* RZS1 is higher than the PHB depolymerase activities reported in *M. paraoxydans* [2], *Penicillium expansum* [19] and *Penicillium* sp. DS9701-D2 [34]. Sayyed and Chincholkar [17] reported the production of higher amounts of PHB in *A. faecalis* and it is known that the PHB depolymerase activity is proportional to the amount of PHB accumulated by the cell. Therefore the higher PHB depolymerase activity is because of the accumulation of more amounts of intracellular PHB by *A. faecalis* RZS1.

Agro-waste like corn waste, rice straw, and wheat straw, is the rich source of carbon but deficient in nitrogen and are the best substrates for PHB production, as PHB production requires carbon-rich but nitrogen-deficient conditions. The results of kinetic models demonstrated a good fit with the model for both biomass and PHB production in *Pseudomonas* sp. RZS1 and *A. faecalis* RZS4. However, *Pseudomonas* sp. RZS1 follows the Monod model more closely as compared to *A. faecalis* RZS4. This indicated the more suitability of *Pseudomonas* spp. for production of PHB from vegetable oils. Production of PHB from vegetable oils offers the opportunity of producing a variety of PHB having different copolymer constituents that may great industrial applications. It also offers greater economic feasibility to enhance the commercial and cost-effective production of PHB.

2. Materials and Methods

2.1 Source of culture

Two bacterial cultures namely *Alcaligenes faecalis* RZS4 and *Pseudomonas* sp. RZS1 used in this study were obtained from the culture repository of PSGVP Mandal's Arts, Science and Commerce College, Shahada, India. These cultures were previously isolated from the local dumping yard and identified [3].

2.2. Culture media and growth conditions

PHB accumulation is a two-stage process; first phase of cell growth followed by second phase of PHB accumulation. For cell the carbon-rich growth medium (CRGM) containing (g/L) glucose, 20; (NH₄)₂SO₄, 2.0; KH₂PO₄, 13.3; MgSO₄·7H₂O, 12.; citric acid, 1.7; and 1.0 ml of trace element solution; containing (mL/L) FeSO₄·7H₂O; 10, ZnSO₄·7H₂O, 2.25; CuSO₄·5H₂O, 1.57; MnSO₄·5H₂O, 0.5, CaCl₂·2H₂O, 2.0; Na₂B₄O₇·10H₂O, 0.23; (NH₄)₆Mo₇O₂₄, 0.1; pH was set to 7.2 [34]. The log phase cultures (10⁻⁵ cells/mL) of *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 were separately grown in CRGM at 30 °C at 120 rpm for 24 h. After incubation, broths were centrifuged at 10000 rpm for 10 min and the cell mass of these cultures were grown at 30 °C at 120 rpm for 48 h in the PHB accumulation nitrogen deficient medium (NDM) containing (g/L), Na₂HPO₄, 3.8; KH₂PO₄, 2.65; NH₄Cl, 2.0; MgSO₄, 0.2; fructose, 2.0; EDTA, 5.0; ZnSO₄·7H₂O, 2.2; CaCl₂, 5.45; MnCl₂·6H₂O, 5.06; H₃BO₃, 0.05; FeSO₄·7H₂O, 4.79; NH₄Mo, 24.4; CoCl₂·6H₂O, 1.6; and CuSO₄·5H₂O, 1.57 [35]. Following the incubation broth were centrifuged at 10000 rpm for 10 min and the cell mass was subjected to the extraction and estimation of PHB.

2.3 Source of agro-wastes and vegetable oils

Agro-wastes such as corn waste, rice straw, and wheat straw were obtained from local farms. These substrates were hydrolyzed with 1 N HCl. Crude vegetable oils such as mustard, corn, sesame, and soybean oils were procured from the local market.

2.4. Screening for PHB production

A. faecalis RZS4 and *Pseudomonas* sp. RZS1 were screened for PHB accumulation by a viable colony method employing the use of Sudan Black B on nutrient agar [14]. For this purpose, each culture was grown (spot inoculation) on nutrient agar at 30 °C for 24 h followed by flooding with Sudan Black B solution (0.02% in ethanol) for 30 min and observed for the appearance of dark black-blue colored colonies [3]. The excess stain from colonies was removed by washing with ethanol (96%).

2.5 Detection of PHB by Sudan black B staining

The intracellular accumulation of PHB in the cytoplasm of isolates was detected by Sudan

black B staining of cells grown in NDMM [36]. For this purpose, a smear of cells from NDMM was stained with 0.3% (w/v) Sudan Black B solution for 15 min followed by de-staining with alcohol (50% v/v) and counterstaining with safranin solution (0.5%, w/v). The slides were then washed; air-dried and observed for the appearance of black-colored PHB granules in a red-colored cell under a bright field microscope with a micro image projection system (Model MIPS, Olympus, Mumbai, India) under an oil immersion lens.

2.6 Production of PHB

PHB accumulation occurs in two stages, during the first stage cell growth occurs in the CRGM followed by the second stage of PHB accumulation in NDM. For this purpose, 1% inoculum of *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 were individually grown in each CRGM at 30 °C at 120 rpm for 24 h followed by centrifugation of broth at 10,000 rpm for 15 min to separate cell biomass. The cell biomass was washed with sterile distilled water and grown at 30 °C at 120 rpm for 48 h in each NDM followed by centrifugation and qualitative and quantitative detection, extraction, and estimation of PHB [37].

2.7 Growth and PHB accumulation as a function of time

For checking the optimum growth and PHB production as a function of time, log phase culture (5×10^5 cells/mL) of *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 were grown in NDM at 30 °C for 48 h at 120 rpm. Samples removed after 6 h intervals were assayed for measurement of growth and PHB [37].

2.8 Kinetic study of growth and PHB formation

A mathematical model of the growth kinetics and PHB production was developed. It was assumed that the micro-organisms follow Monod Growth kinetics for growth and Luedeking and Piret equation for product formation. Fructose was taken as the rate-limiting substrate. The rate of formation of biomass and product and the rate of substrate consumption can be given by the following equations-

$$\begin{aligned}\frac{dx}{dt} &= \frac{\mu_{max}x}{k_s + S} \\ \frac{dP}{dt} &= \alpha \frac{\mu_{max}S}{k_s + S}x + \beta x \\ \frac{dS}{dt} &= -\frac{\mu_{max}S}{Y_{x/S}(k_s + S)}x - \frac{1}{Y_{P/S}}\left(\alpha \frac{\mu_{max}S}{k_s + S} + \beta x\right)\end{aligned}$$

Where,

x is biomass, μ_{max} is the maximum specific growth rate,

k_s is half-velocity constant,

S is the substrate concentration,

P is the product concentration,

α and β are the empirical constants,

$Y_{P/S}$ is the yield of product per unit mass of the substrate consumed and

$Y_{x/S}$ is the biomass formed per unit mass of substrate consumed.

These equations were simultaneously solved using the 'odeint' function of the 'scipy' module of Python 3.7. The values of the constants were predicted by fitting the experimental data of biomass growth and PHB formation to the equations.

2.9 Extraction and estimation of PHB and measurement of growth

PHB is accumulated inside the cell therefore; extraction of PHB required the lysis of PHB cell accumulating cells. Such cells become fragile and are easily lysed. For extraction of PHB, the cell mass from NDM was lysed by using sodium hypochlorite at 37 °C for 1 h, the content was centrifuged at 10,000 rpm for 10 min, the precipitate was washed with distilled water and PHB from cell lysate was extracted with a mixture of acetone: ethanol (1:1). The extracted PHB was precipitated in chloroform. The precipitate was dried at room temperature to derive the PHB in powder form [12]. PHB extract dissolved in a small amount of chloroform was measured by the gravimetric and spectrophotometric method [38]. In the gravimetric method, the PHB content of chloroform extract was determined as a difference in the weight of the sample before drying and after drying to constant weight. It was expressed as mg % of dry cell mass or g l⁻¹ of the medium.

In spectrophotometric methods, the PHB content of the extract was quantitatively estimated according to the method of Law and Sleepecky [37] that converts PHB into crotonic acid. For this purpose, PHB extract (10-100 µg) was prepared in chloroform; the chloroform was evaporated by gentle heating at 40 °C. A 10 ml of concentrated sulfuric acid was added in each tube and incubated at 100 °C for 10 min. The solution was cooled to 28 °C and the amount of PHB present in the sample and cell mass were measured at 254 nm and 620 nm, respectively [37].

2.10 Production of PHB from agro-waste

To evaluate the effect of agro-waste like corn waste, rice straw, and wheat straw on the production of PHB, these substrates were acid hydrolyzed with 1 N HCl and were individually added @ 20 g l⁻¹ as the sole carbon source in each NDM. Each NDM was separately grown with log phase culture of *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 at 30 °C for 48 h at 120 rpm. After the incubation, cell mass separated by centrifugation was measured, and PHB was extracted and estimated [37].

2.11 Production of PHB from crude vegetable oils

Vegetable oils are a good source of a wide variety of free fatty acids and thus provide good substrates for the biosynthesis of a diverse variety of PHB. These PHB with different copolymer constituents may have great commercial potential. For this reason, PHB production from vegetable oil was undertaken in NDM. For this purpose 20 g l⁻¹ each of vegetable oils such as mustard, sesame, and soybean oil were separately added as a carbon source in each NDM after the inoculation and incubation, measurement of cell growth and extraction and estimation of PHB were performed as described earlier [37].

2.12. Characterization of PHB extracts

2.12.1. UV-Vis spectrophotometry

For this purpose, PHB extract (5.0 mg) dissolved in 5.0 ml of chloroform was scanned between 190-1100 nm on a UV-Visible spectrophotometer and observed for the absorption maxima characteristic of PHB [37].

2.13. Assessment of biodegradation of PHB

2.13.1 Plate assay

Plate assay is a semi-quantitative estimation method used to screen polymer degrading organisms and for measuring the biodegradability of a polymer. To assess the biodegradability of PHB extract produced by *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1, the log

phase cultures of both organisms were separately grown (5×10^5 cells/mL) on NDMM containing PHB extract (as the carbon substrate) at 30 °C for 48 h and observed for the formation of clear zone around the colony as an indication of utilization/ biodegradation of PHB [12].

2.13.2 Shake flask method

Biodegradation of PHB was performed on PHB film prepared from PHB extract. For this purpose, PHB powder (0.15 gm) dissolved in chloroform (50 mL) was added into two petri-plates and incubated at 30 °C for 10 h. PHB film was obtained by evaporation of chloroform at 50 °C. PHB films were separately added as the sole carbon substrate into two NDM, one flask was grown with log phase culture of *A. faecalis* RZS4 and another flask was grown with *Pseudomonas* sp. RZS1 at 30 °C for 8 days under shaking (120 rpm), samples withdrawn after 24 h interval were analyzed for qualitative estimation (weight loss) and quantitative estimation according to the method of Law and Slepecky [37].

2.13.3 Assay of PHB depolymerase enzyme

In enzyme assay, the polymer is exposed to a pH-controlled system containing PHB depolymerase, which mobilizes PHB into an oligomer or monomers. For this purpose, two NDMM individually grown with *A. faecalis* RZS4 and *Pseudomonas* sp. RZS1 at 30 °C for 8 days were centrifuged (10,000 rpm for 10 min) and the supernatant was assayed for PHB depolymerase activity. The reaction mixture comprising of 100 µg/mL PHB (substrate), 2 mM CaCl₂ and 0.5 ml culture supernatant was incubated at 30 °C for 10 min and decrease in absorbance due to biodegradation of PHB was measured at 650 nm. One unit of PHB depolymerase was defined as the amount of enzyme that decreases the absorbance by 0.1/min [38].

2.14. Statistical analysis

All the experiments were performed in **five replicates** and the average values of five replicates were statistically analyzed by **one-way** analysis of variance (ANOVA) **followed by Turkey's** test. Mean values of $P \leq 0.05$ were considered statistically significant [39].

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Author contribution

RZS, conceptualization, supervision and administration; SSS and SJW, Methodology; MTR, MFAA, KJ and VS Formal analysis and SH and HEE, Writing - review and editing.

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