PRODUCTON OF BIOPOLYMER FROM FOOD WASTE

Dissertation submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

By

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DECLARATION

I hereby declare that the work reported in the M.Sc. dissertation entitle "**PRODUCTION OF BIOPOLYMER FROM FOOD WASTE"** submitted at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, isa authentic record of my work carried out under the supervision of Dr. Garlapati Vijay Kumar, Dept. of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh-173234, India. I have not submitted this work elsewhere for any other degree or diploma.

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Date-

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M.Sc. dissertation entitle "**PRODUCTION OF BIOPOLYMER FROM FOOD WASTE**" submitted by Dheeraj Kumar (207809) at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, is bonafide record of his original work has not been submitted elsewhere for any other degree or diploma.

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Abbreviations

MSW -------Municipal Solid waste

HA ------ ---Hydroxyalkanoate

HB –-------- Hydroxybutyrate

LDPE ------Low density polyethylene

MCL -------Medium chain length

SCL---------Short chain length

MMC ------ microbial mixed cultures

PHB ------ polyhydroxybutyrate

PHAs------ polyhydroxyalkanotes

P(3HB) ----poly(3-hydroxybutyrate)

TOC – total organic carbon

L.B---------- Luria Bertani broth

RPM-------- rotation per minutes

UV----------ultraviolet

UNEP------united nation environment programme

CAGR-----compound annual growth

ABSTRACT

Polyhydroxyalkanoates (PHA) are biodegradable polymer and eco-friendly thermoplastic, which are accumulated as carbon and energy storage materials in various bacteria.

In this study, bacteria sample were taken from microbiology lab of Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, for the purpose of production of biopolymer using food waste as a C-source. To obtain PHA from *Ralstonia Eutropha,* food waste used as carbon-source at different incubation period. All synthesized intracellular inclusion during growth on starch carbon-source. *Ralstonia Eutropha* cultivation were proven promising for PHA production. The inclusions were predominantly identified as Polyhydroxyalkanoates using and spectrophotometer. The best result was 24 hours incubated *R. eutropha* with food waste, PHA crystal was achieved which was grown in 20g/l foo waste powder.

Keyword

Food waste, Polyhydroxyalkanoates, Biodegradable, Sudan black B, Nile Blue A, Pure culture, culture, incubation, eco-friendly, cost-effective.

CHAPTER 1

Introduction

==========

1.1 Food waste

Food waste is one of the major problems for the environment. Due to lack to government plan, maintenance and government policies food waste mix with Municipal Solid Waste (MSW) and it discarded to open dump or landfill that results increase the pile of food waste at duping site. Major other problem also occurs at the open dump or land fill mainly due to food waste that may cause sevier disease. Food waste is mainly occurring by the action of human being, when edible food items go unconsumed resulting another pile of food waste. As methane gas, leachates and other harmful gases released from the waste which is not good for environment. According to United Nations Environment Programme (UNEP) WRAP, 5.3 billion tonnes food available for human consumption and 931 million tonnes of food waste that is 17% of available food for human consumption. (Hamesh Forbes (WRAP), et al., 2021). it is one of the largest parts of the municipal solid waste (MSW) (E, et al., 2011). Table 1. Globally 8-10% of greenhouse gas emissions are associated with food waste that is not consumed by humans (Hamesh Forbes (WRAP), et al., 2021). Mostly food waste in household activity which account for 61% then the food service and retailers 26% and 13% respectively. figure1

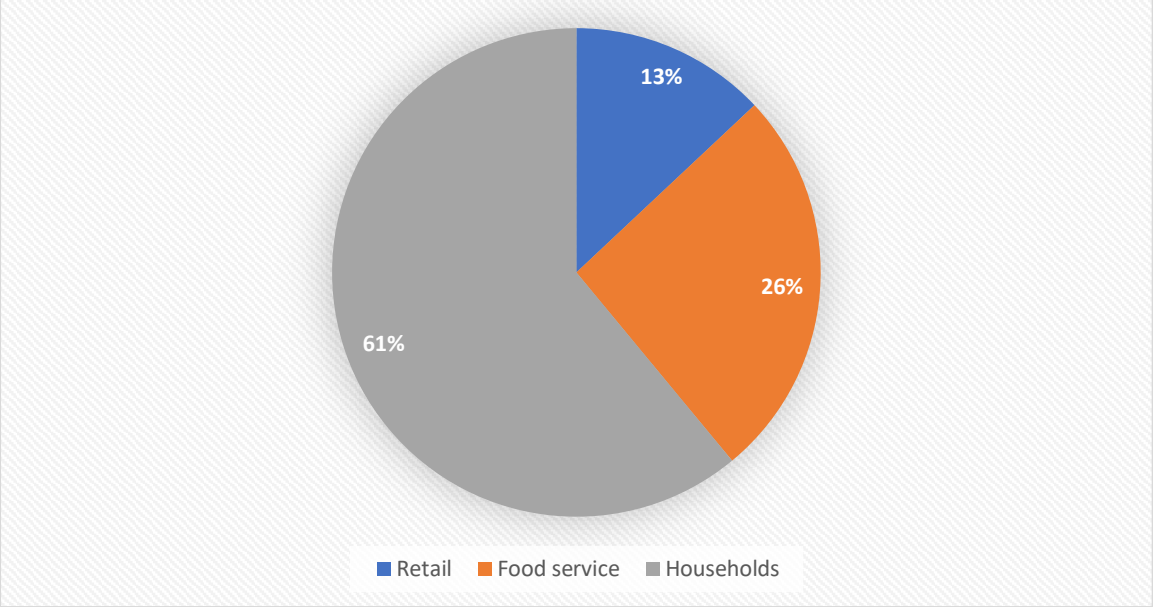

Figure 1 source of generated waste and % of total waste

According to UNEP globally annual per capita food waste is 121kg/capita/year. Figure 2. In India, household food waste approximately is 50kg/capita or 68,760,163 tonnes in a year. (Hamesh Forbes (WRAP), et al., 2021). Cereals are roughly 4.65 percent to 5.99 percent, pulses are 6.3 percent to 8.41 percent, oil seeds are 3.08 percent to 9.96 percent, fruits and vegetables are 4.58 to 15.88 percent, milk is 0.92 percent, meat is 2.71 percent, and chicken meat is 6.74 percent.(Hamesh Forbes (WRAP), et al., 2021)g.

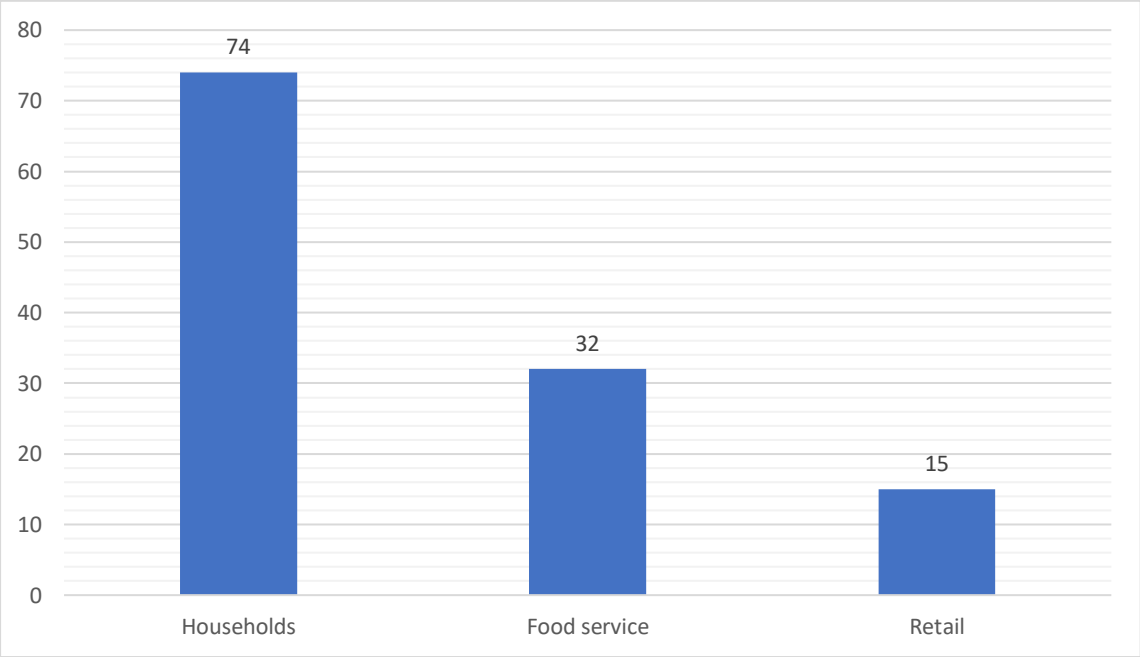

Figure 2 Annual per capita food wastage (GLOBALLY) :121 kg

The European Commission is considered declaring 2014 "Food Waste Prevention Year." The European Parliament passed a resolution seeking to cut food waste in the EU in half by 2025. Food waste such as vegetables, rice, pulses, cereals, roots and tubers, oilseeds milk, eggs. Meat, seafood etc. that food waste contains 74-90% moisture, 80-97% volatile solid to total solid and 14.7-36.4 carbon-nitrogen ratio. (Zhang, et al., 2007) But at the end all the food waste got dumbed in landfill, incineration while these methods are not suitable for treating food waste, that may cause contamination of air, water, and soil due to its odour, leachate and quick decompositing nature during the collection, transportation and storage. As food waste contains moisture, volatile solid to total solid, carbon, nitrogen and other gases that can be use in different ways such as food wase can use for bio fuel production, can use for bio fertilizer, can

use for Nutraceuticals and bioactive compounds, can be used for biopolymer production using different bacteria by fermentation and other methods. In this thesis we discussed about as title says -Biopolymer production from food waste. As food waste is much cheaper C-source than other commercial media. Biopolymers are the polymer which is derived by a cell or microorganism. Biopolymers are better alternative of synthetic polymer. Synthetic polymers take much longer time to decompose whereas biopolymer takes less time to decompose. Due to less expenditure on production of synthetic polymer and production of biopolymer is costly thus companies prefer synthetic polymer over biopolymer. But since last few decades pollution increasing drastically and government of several countries ban on production of synthetic plastic. After the ban on synthetic biopolymer and bioplastic, demands of bioplastic and biopolymer increased. Market value of PHAs is not good but now it is attracting the people and increasing in interest growing of PHAs but is increasing. (Europeanbioplastics, 2020) figure 3. In India biopolymer market is grow at a CAGR of 23.91% by 2025. In 2019 it is of US$208.475 and expected to reach at US$754.648 by 2025. Many economic entities not at all investing in research and development to develop innovative techniques that might lower the cost of producing biopolymers. In June 2020, Lygos, Inc. of Berkley, California, and Praj Industries Ltd. of Pune, India, signed a Memorandum of Understanding for PLA production. In September 2019, Total and Carbion formed a 50/50 joint venture with Konkan Speciality Poly Products Pvt Ltd. had announced its intention to jump in the biopolymer market of India. (intelligence, 2021)

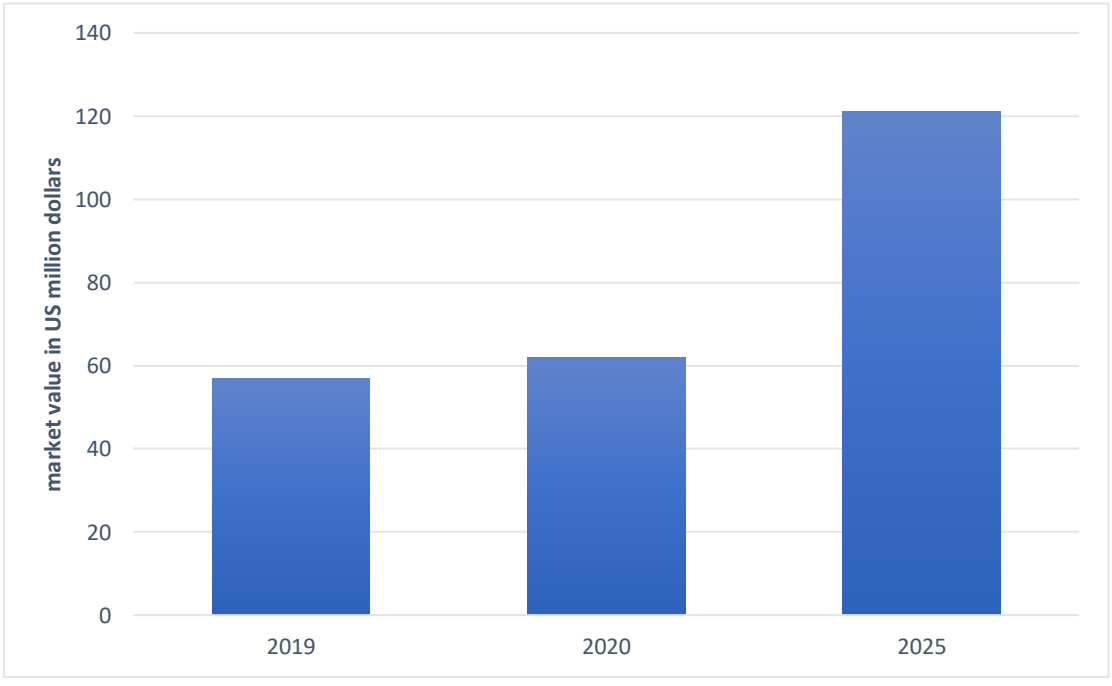

Figure 3 Market value of PHA since 2019-2020 till 2025

Table 1 Country-wise annual per capita food wastage at households.

Country	Kg/capita/year
Worst Five	
Nigeria	189
Rwanda	164
Greece	142
Bahrain	132
Malta	129
Other Countries	
Israel	100
UAE	95
South Korea	71
China	64
Russia	33
G-7 Countries	
France	85
Canada	79
UK	77
Germany	75
Italy	67
Japan	64
USA	59
South Asian Countries	
Afghanistan	85
Bhutan	79
Nepal	79
Sri Lanka	76
Pakistan	74
Maldives	71
Bangladesh	65
India	50

Biopolymer production from food waste become hot topic among the researcher because of the properties of biopolymer and the use of cheaper feed for the microorganism. Using food waste for the purpose of production of Biopolymer it will deal with the two environmental problem one is decreasing the pile of food waste and major issue of synthetic plastic. Biopolymer can be use in clothing fabrics, medicines, pharmaceutical, industrial plastics, biosensor, packaging, food industries, water treatment, chemicals, and in cosmetics. (Sanchez-Vazquez, et al., 2013)

1.2 Biopolymer

Plastic that we use every day, that is made from various chemical which is not suitable for our environment. That's why we need alternative of plastic i.e., biopolymer or bioplastic. Production of Biopolymer from food waste that will be beneficial in financial and environmental. When plastics are dumped into landfills, it takes longer time to degrade due to absence of sunlight in landfills, whereas biopolymer degrades in landfill environment by several soil bacteria. (Grage, et al., 2009). Due to its high production cost, high bacterial feed, Currently, biopolymers are not widely used. Unaffordable bacterial feed can be avoided by using food waste as bacterial feed. Cheaper feed for the microorganism, production of PHA would be easier and easily available to the consumer. Biopolymer that are produced from a cell or a whole microorganism under certain condition. Biopolymer can use as alternative for plastic because of Biopolymers have similar properties like conventional petroleum-based plastic but bioplastics are biodegradable and eco-friendly whereas conventional petroleum-based plastics are not biodegradable and nor eco-friendly.

Different type of biopolymer derived from different source such as PGA (Polyglycolide) derived, PLA (Polylactic acid), PLGA (Poly-lactide-*co*-glycolide), PCL (Polycaprolactones), PBS Poly (butylene succinate), PPDO (Poly-p-dioxane), poly-carbonate, polyamide, and polyesteramides, polyurethanes, polyanhydrides, and vinyl polymers are derived from petroleum resources, PHA (Polyhydroxyalkanoates) and PHB (Polyhydrobutyrate) are biodegradable polymer which is derived from microbial cell by fermentation.

1.3 PHA

PHA is natural occurring polymer that accumulate inside the microorganism, its molecular weight ranging from 5×10^4 to 2×10^6 Da. (Anderson & Dawes, 1990). PHA gained more

popularity in short term because of its properties. Due to its expensive potential industrial application, it gained the full attention from the researchers. PHA are one of the biodegradable biopolymers which is mainly produced by several bacteria as an intra-cellular back-up material. More tha 300 bacteria from the family of, *Halobactericeae,* are some o of the then are involve in PHA synthesis. (Suriyamongkol, et al., 2007) PHAs are divided into three groups based on the length of their chains. PHAs with a maximum chain length of five carbon atoms are known as short chain length (scl)PHAs. PHA contains 6-14 carbon atoms in its medium chain length (mcl). The chain is long and contains more than 14 carbon atoms. Table 2 Dur to a smaller number of carbon atom, Scl-PHA can be used to produce rigid plastics whereas in mcl-PHA has more carbon atom than Scl-PHA, mcl-PHA can used in flexible material. Microorganism can produce variety of PHA monomer subunits, over 155 unique monomer subunits is confirmed in a report. (Agnew & Pfleger , 2013). *Ralstonia Eutropha* was chosen for the purpose of production of PHA in this protocol because their internal structure is more over similar to the PHA and *Ralstonia Eutropha* has significant potential. It's also good for bioremediation since it can breakdown a lot of chlorinated aromatics. Compounds and pollution caused by chemicals. Ralstonia eutropha can also metabolise heavy metals, making it a potential option for polymer synthesis research. *Ralstonia eutropha,* it also uses in making other useful chemicals and fuel with a little bit genetic modification.

PHA first discovered by Lemogine in 1920, it is a family of naturally occurring biopolymer which is synthesized or accumulated by several microorganism. (Anderson & Dawes, 1990). Generally, PHA molecule is made up of 600-35,000 (*R)*-hydroxy fatty acid monomer units. (Khanna & Srivasta, 2005). A side chain R group, which is commonly a saturated alkyl group, is present in each monomer unit. Having a saturated alkyl group, although it can also take the form of an unsaturated alkyl group, a branching alkyl group, or a substituted alkyl group, which is mostly seen in rare cases.. (Lu, et al., 2009). Figure 4

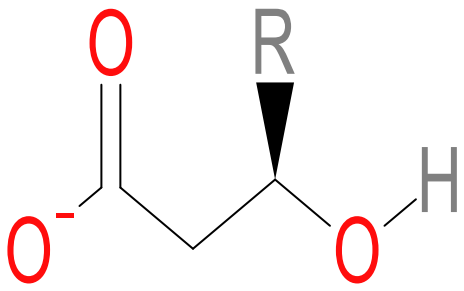

Figure 4 poly[(R)-3-hydroxyalkanoate]

Several microorganisms can produce PHA. Actually, PHA is a carbon storage microorganism which is used by microorganism as alternative source of fatty acid. Under stress condition microorganism use it for their survival, it is mechanism for their survival. (Singh Saharan, et al., 2014)

Table 2 Types of PHA, based on carbon number

***R* group**	**Carbon No.**	**PHA polymers**
Methyl	Carbon- 4	PHB Poly(2-hydroxybutyrate)
Ethyl	Carbon- 5	PHBV Poly(3-hydroxyvalerate)
Propyl	Carbon- 6	PHBHHx Poly(3-hydroxyhexanoate)
Butyl	Carbon- 7	Poly(3-hydroxyheptanoate)
Pentyl	Carbon- 8	Poly(3-hydroxyoctanoate)
Hexyl	Carbon- 9	Poly(3-hydroxynonanoate)
Heptyl	Carbon- 10	Poly(3-hydroxydecanoate)
Octyl	Carbon- 11	Poly(3-hydroxyunddecanoare)
Nonyl	Carbon- 12	Poly(3-hydroxydodecanoate)
Decyl	Carbon- 13	Poly(3-hydroxytridecanoate)
Undecyl	Carbon- 14	Poly(3-hydroxytetradecanoate)
dodecyl	Carbon- 15	Poly(3-hydroxypentadecanoate)
tridecyl	Carbon- 16	Poly(3-hydroxyhexadecanoate)

1.4 Properties of PHA

PHAs (Polyhydroxyalkanoates) are one of the attractive biopolymers since last few years due to their properties and it is similar to petroleum-based polymer. PHA are biodegradable thermoplasticity polymer and it can degrade in different environments, having less functional group. PHAs are hydrophobic in nature, versatility in their processing. PHA are carbon-based polymer. PHA are good resistant to UV rays. PHA are one of the polymers that have high degree of polymerization and stiffness.

PHA exist only in D configured it is not existed in L-configured because of the stereospecifity of the biosynthetic enzyme. PHA has 60-70% crystalline. Due to its intrinsic brittleness nature, it has limited use. its melting temperature is 165-180°C and thermally decomposes at 270°C.

CHAPTER 2

Review of literature

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2.1 PHA synthesis: Sources and utilized Microbial strains

Table 3 PHA from different sources and microorganisms

Food waste sources	strain	biopolymers	Biopolymer percentage	References
Crab waste	*Lactobacillus sp.* B2	Polyhydroxyalkanoates	34	(Flores-Albino, et al., 2012)
Prawn shell	*Cocultivation of lactococcue Latis and Teredinobacter turnirae*	Polyhydroxyalkanoates	64.5	(Aytekin & Elibol, 2009)
Rice straw	*Bacillus firmus*	Polyhydroxybutyrate	89	(Ali & Vidhale, 2013)
Wheat bran and rape seed meal	*Cuprividua nector*	Polyhydroxybutyrate	78.9	(Kachrimanidou, et al., 2016)
Spent coffee grounds oil	*Cuprividua nector*	Polyhydroxybutyrate	89	(Obruca, et al., 2014)
Corn oil	*Pseudomonas*	Polyhydroxyalkanoates	35.63	(Chaudhary , et al., 2011)
Waste frying oil	*Cuprividua nector*	Polyhydroxybutyrate		(Verlinden, et al., 2011)
Whey	*Thermus thermophiles HB8*	Polyhydroxyalkanoates	35.6	(Pantazaki, et al., 2009)
Molasses	*Pseudomonas*	Polyhydroxyalkanoates	20.63	(Chaudhary , et al., 2011)
Cheese-whey	*Methylobacterium sp.*	Polyhydroxyalkanoates	51	(Obruca , et al., 2011)
Cheese-whey	*H. pseudoflava*	Polyhydroxyalkanoates	40	(Koller, et al., 2007)
Cheese-whey	*B. megaterium*	Polyhydroxyalkanoates	67	(Nath, et al., 2008)
Soy bean and rapeseed oil	*Cuprividua nector H16*	Polyhydroxybutyrate	79	(Taniguchi , et al., 2003)
Juice from oil palm (pressed)	*Cuprividua nector*	Polyhydroxybutyrate	30	(Zahari, et al., 2012)

As reported in literature PHA can be synthesized from several type of C-source such as agriculture waste including rice husk, wheat husk, wheat bran, coffee bran and etc. industrial waste such as unused milk, waste water from the dairy, juice industries, cooked food such as chapati, rice, cereals, several veggies and cooking oil. Here we discussing for the purpose of production of cooked and cooked food. Different type of food waste and several microorganisms which is used in production of PHA. Mentioned in Table 3.

2.2 PHA from dairy wastes

PHA can be produce from the dairy, and its unused food: food waste. In the dairy there are several types of waste that contains suspended solids, trace organic (oils, fats, minerals, phosphate and grease) soluble organics, and other necessary ingredients that can be used for the purpose of production of PHA with the help of microorganism. (Sarkar , et al., 2006). For the better result of PHA shaker speed should be 150rpm, temperatures should be 37°C and butter milk act as a C-source at pH -7 during fermentation. (Mehta, et al., 2017). Several C-source can be used for the purpose of production for the PHA production but from the dairy waste, buttermilk was used as it the low-cost C-source from the dairy industry for the purpose of production of PHA. (Mehta, et al., 2017)

2.3 PHA production from sugarcane industry waste

Sugarcane is the world's largest cash crop. Brazil is the leading country for the sugarcane cultivation, India is the second in number for the cultivation. Sugarcane is the unused fibrous residue of sugarcane stalk which is remain after the extraction of juice and major lignocellulose, inexpensive by product of the sugarcane industry. (Pippo & Luengo, 2013). As reported SCB, cellulose (46For the purpose of production of PHA from the sugarcane industry waste %, hemicellulose 27%, lignin 23%, and ash content is 4%. (Pippo & Luengo, 2013), Due to the fibrous nature of SCB, is hard and slow utilization by the bacteria. To make more easily utilize by the microorganism SCB converted into powered form. Being all these properties of SCB has potential to produce PHA. *Halococus salifodinae, Haloferax volcanii, Haloarcula japonica and Hgm borinquense* show best result. (Salgonkar & Braganca , 2017). Concentration of SCB (Sugarcane Brash) hydrolysate, inhibit the growth of microorganisms. The higher concentration of SCB hydrolysate, 50%,75% and 100%, that was determined by the culture grown on NSM (Nutrient Sporulation Medium) agar plate. For the best result sugarcane bran dried, cut into small pieces and converted into powered form. (Salgonkar & Braganca , 2017).

2.4 PHA from fruits and vegetable wastes

Waste from juice industry, brewery industry, residue from food processing industries and many vegetables processing waste from industries are nearly tough to proper discard such biomass. Due to not proper discard of these waste, it creates pollution and environmental problem. Surprisingly, all these can be used for the purpose of production of PHA which is already reported by (Di Donatoa, et al., 2014). For the purpose of production of PHB from the food waste *Haloterrigena hispanica, Geobacillus thermoleovorans subsp. Stromboliensis, Geobacillus thermantacitucus,* bacterial culture was grown using complex and minimal media as described by (Di Donato, et al., 2011). Before use of fruits and vegetables, it was dried under vacuum, chopped into smaller size and converted into powdered form. Batch fermentation and dialysis fermentation was two different process was tested that support the growth of microorganism using fruit and vegetable waste. A report by (Romano, et al., 2007), that described about the application of PHA from the vegetable waste for the medical, medicine and packing of different cosmetics that reduce could reduce the cost of packaging.

2.5 PHA from poultry waste

About 7.7×10^8 kg of feathers produce annually by the poultry farm (Taskin, et al., 2011). Feathers, wings and vestigial organs of chicken can be used as C-source by *Cupriavidua nector* H16 for the purpose of production of PHA. It could decrease the cost by 50% for the purpose of production of PHA. (Palareti , et al., 2016). Chicken feathers were washed and dried and sterilized for 30 min at 121°C. (Taskin, et al., 2011). Chicken feather waste fermented with the *Pseudomonas Putida* and nitrogen-limited mineral media for the purpose of production of mcl-PHA and the yield was $1.42gL^{-.}$ (Pernicova , et al., 2019). Non-treated chicken feathers use as C-source for the growth of *Pseudomonas. p* using minimal salt medium for 7days at 30°C with shaking at 180rpm. 10g L^{-1} Poultry litter supplement with 10% CO_2 fermented with *Cyanobacterium, Nostoc muscorum Agardh,* and the yield was 144.42mg L^{-1} of PHB. (Bharti & Mallick , 2015). For 10 days of incubation with the *Cyanobacterium* show rise in biomass yield with up to the 10 % CO_2. 1.12 g L^{-1} and 744 mg $L^{-1,}$ 65% of dry cell wt. of PHA under the optimal control biomass was recorded by (Bharti & Mallick , 2015).

2.6 PHA from fish waste

Globally about 174.6 million metric tons fish produced in 2020 and about 35% of production is lost or wasted. (FOA, 2020). Fish waste generated depending on various types of fish, product and processing techniques. Heads, fins, skin, frames, trimming are the major fish waste

from fisheries and aquaculture. These cheap substrates can be used as carbon or nitrogen source for the purpose of production of PHA and it also lessen the problem of the environments. (Mohapatra , et al., 2017) The production of fish meal and fish oil for farmed animals. (Ghaly, et al., 2013), (Caruso, 2016). Nitrogen and C-source from fish sauce and mixture of waste fish oil, glycerol fermented with Halophilic bacterium, *Salinivibro sp.* Use for the purpose of production of PHA. (Van Thuoc, et al., 2019). In a report of (Van Thuoc, et al., 2019), fish oil and glycerol (1:1, w: w) is used as nitrogen source, ferment with bacterial strain *Salnivibrio sp.* For 48 hours at 180 rpm rotary shaking, 30°C temperature, pH 6.5 grown in LB medium and the result was 5.8 g/L.

2.7 PHA from Rice mill and other malt house dissipate

Bran, husk, straw from the rice and wheat are waste from the mills. As it is cost-effective and easily available is mainly use for cattle feed and used in plantation for maintaining moisture in soil and after sometime it is act as manure for the plants. Waste from mills is mainly consist of lignin, cellulose, hemicellulose, all are cheap C-source and can be used for bacterial fermentation. About 0.22g P^{-1} polymer yield from the wheat straw fermentation with the *Bukholderia sacchari*. Reported by (Gesario, et al., 2014)

2.8 PHA from coffee spent grounds (SCG)

In march 2022, 11.79 million bags of green beans exports globally, which is higher than previous years. Total green beans production was 11.48 million bags, which is grow upto 2.6%. (Organization, 2022) and about 167.26 million bags consumed in the 2020-21 which is increased by 1.9% i.e., 164.13 million bags (Organization, 2022). In the coffee industry, spent coffee grounds is oiled (9-15%) and is considered as waste. Actually, coffee grounds are the by- product of coffee processing. (Al-Hammare, et al., 2012). Coffee oil contain high percentage of oil which is extracted form coffee grounds and oil in coffee is like waste cooking oils, animals and other biomass residue. (Shi & Bao, 2008). *Bukholderia cepacia*, *C. nector* H16 was used for the purpose of production of PHA by (Obruca , et al., 2014) with SCG and the yield content was 14.2g biomass and PHA content of 70%. Due to its high antioxidant content, it has higher stability and less pleasant smell and less expensive substrate for PHA production. But, due to its foaming nature SCG is not mainly used for PHA production.

CHAPTER 3

Materials and Methods

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3.1 Production scheme of PHA from food waste.

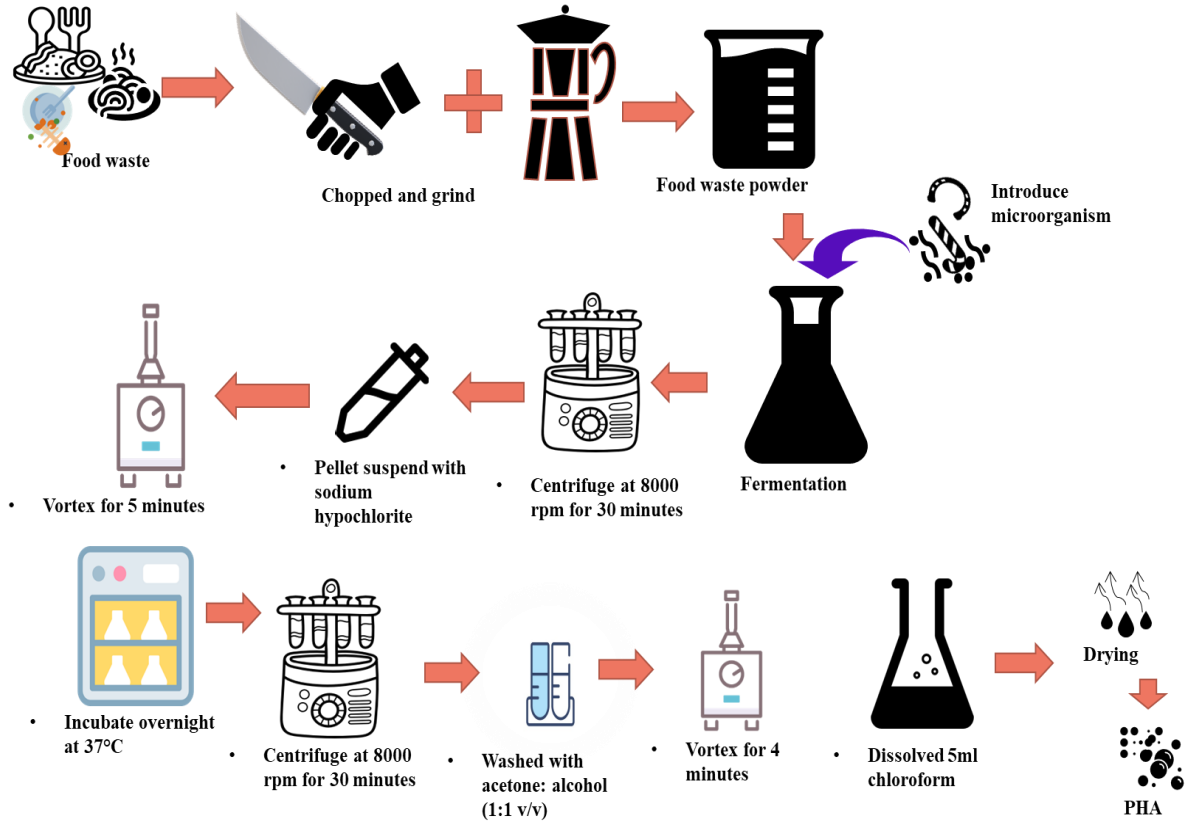

Figure 5 Process of PHA production form food waste.

3.2 Preparation of powder from food waste

Food waste including cereals, rice, roti, paneer, and different vegetables were collected from the mess of Jaypee University of Information Technology Waknaghat, Solan, Himachal Pradesh. Food waste was washed several times to remove oil from food waste. After removal of oil food washed chopped into small pieces and washed again. For removal of water from food waste, it was kept in oven for 10-12 hours at 80°C. After 10-12 food waste was free of oil and water, it was kept at room temperature, allow them to cool for 1-2 hours. Blending was performed at maximum setting for fine powder out of food waste. Food waste powder was

stored in a jar and kept away from the sunlight. Food waste powder was used as feed for the bacterial culture. Figure 6.

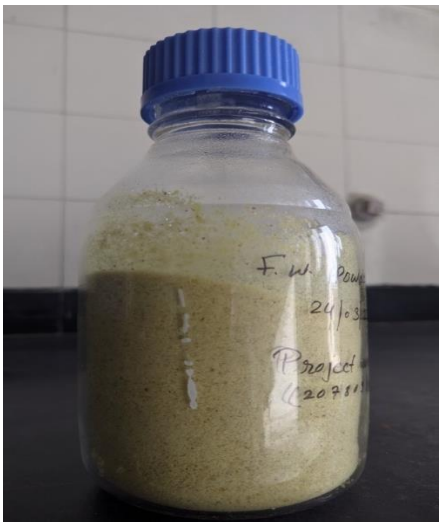

Figure 6 Food waste powder

Figure 7 Bacterial culture (r. eutropha)

3.3 Culturing and screening of bacteria

Bacterial culture was taken from the microbiology lab of Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh. *Ralstonia Eutropha* was the bacterial culture. For the conformation of same bacterial screening was performed by dye and stain. In a first place *Ralstonia Eutropha* was grow in Luria Broth in different test tube at 28°C for 24 hours. After the growth of *Ralstonia Eutropha* in test tube, figure 7, Nutrient Agar was prepared and poured in different petri plate. Grown *Ralstonia Eutropha* was striking at agar plate and

incubated at 28°C for 24 hours. After the growth of *Ralstonia Eutropha* ethanolic solution (0.05% w/v) of Sudan Black B spread over all the colonies and plate kept undisturbed for 30 minutes. After 30 minutes plates were washed with 70% ethanol, colonies were appeared as bluish black. Figure 8. Bluish black colonies indicate PHA positive isolates. After the Sudan Black B isolates *Ralstonia Eutropha* grow again in Nutrient Agar for 24 hours at 28°C. After 24 hours bacterial culture was stained with ethanolic solution (1% w/v) of Nile Blue A solution, then bacterial colonies exposed to UV light for few seconds. Those Colonies exhibiting fluorescence were PHA positive colonies. Figure 9.

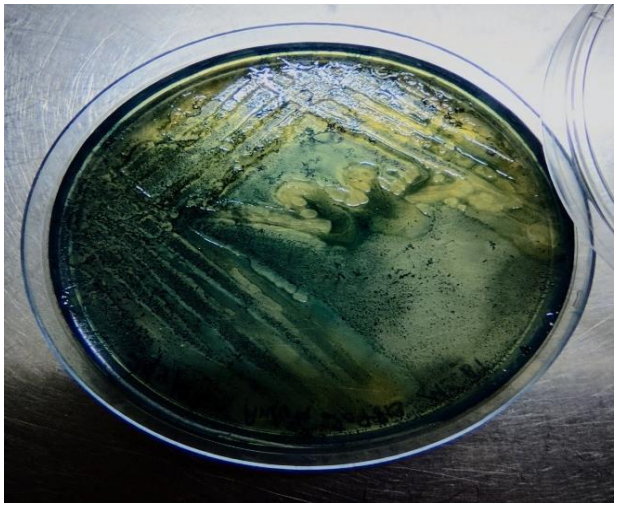

Figure 8 R. eutropha culture after Sudan Black B dye

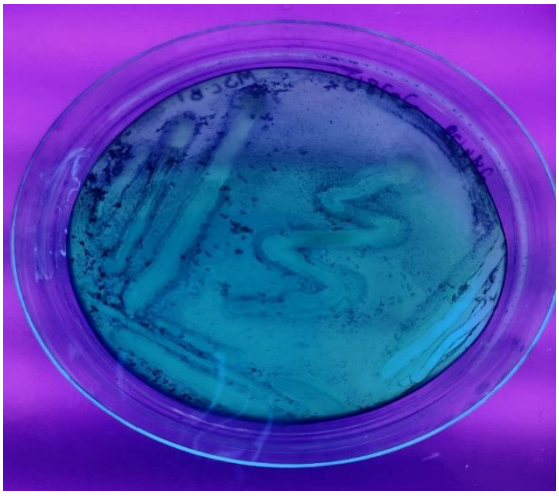

Figure 9 Bacterial culture under UV, For fluorescence, after the Nile Blue A

3.4 Fermentation

20g/L food waste powder was taken and prepared 1Liter broth of food waste powder in 2liter of Erlenmeyer flask. Food waste powder was dissolved properly that was used as C-source for the bacterial colonies and limiting other source of energy. Nile Blue A positive isolates was used as inoculum in same 2Liter of Erlenmeyer flask. After the inoculation 1Liter of broth was divided into 4 Erlenmeyer flask of 250ml and marked as 24 hours, 48hours, 72hours and 96 hours. Broth with bacterial colonies was incubated as marked on the Erlenmeyer flask. Each 250ml Erlenmeyer flask after the assigned hours of incubation grown seen in broth as denser colour or cloudy appearance of broth.

3.5 Extraction

Centrifuge at 800rpm for 45 at 4°C was performed. Pellet was collected and suspended with 5ml of 2% sodium hypochlorite solution. Incubation at 37°C for 30 min after the vortex for 10minutes. Centrifugation was repeated again at 8000rpm for 20 minutes at 4°C. After the centrifugation pellet was washed with acetone: alcohol (1:1) mixture. Then pellet was vortex for 10min. PHA crystals was collected after the applying of 5ml chloroform to the Petri dish, allowed them to evaporate. After the evaporation PHA crystals was scratching from the petri plate *figure* 10, 11, 12, 13 and weighted. Figure15. 10ml of sulfuric acid (95-98) in test tube for 120 min at 100°C in water bath. Kept it to cooldown then it was appeared as dark brown coloured. PHA solubilized in high temperature and converted in crotonic acid. Figure 14

CHAPTER 4
Results and discussions

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4.1 PHA production

Figure 10 PHA crystals of 24 hours

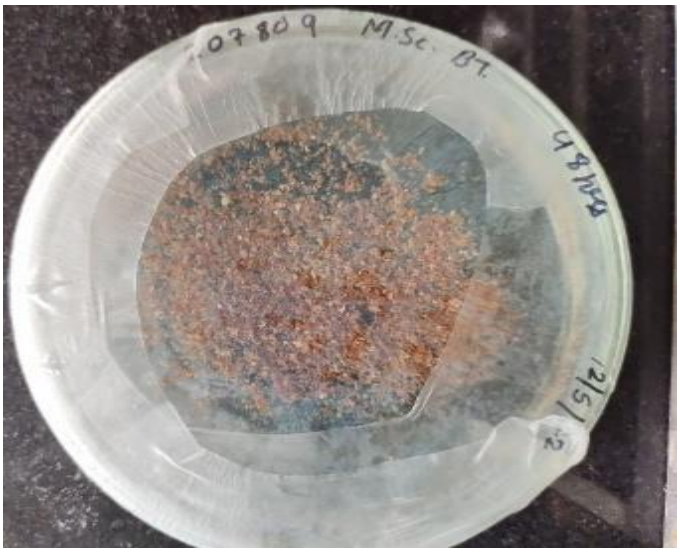

Figure 11 PHA crystals of 48hours

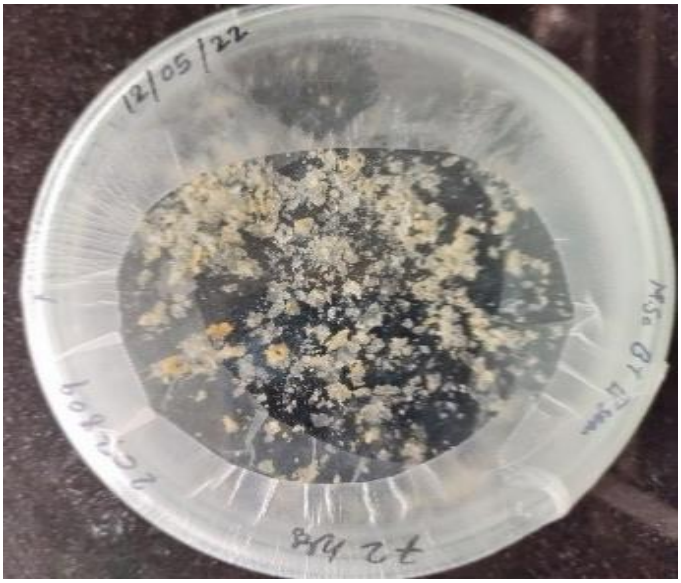

Figure 12 PHA crystals of 72hours

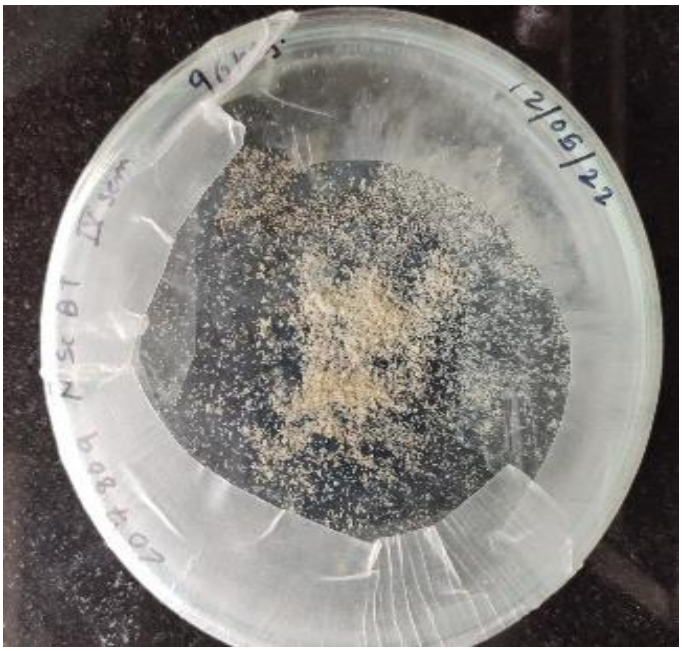

Figure 13 PHA crystals of 96 hours

Figure 14 Dark brown colour appeared after depolymerized

In above figure10, 11,12 and 13, PHA power was obtained from the 24, 48, 72, 96 hours respectively, after the evaporation of chloroform at room temperature. In all obtained PHA crystals 24 hours incubated has more weight than the 48 hours incubated, then 72 hours and the lowest weight of PHA crystals was from 96 hours incubated. Obtained weight is mentioned in figure 15. After the obtained PHA crystals and weighted all the sample then for the conformation test and for estimation of PHA, as suggested by (Slepecky & Law, 1960). Due to dehydration PHA crystal was converted into crotonic acid and appeared brown colourcrotonic after cooling down (Aslim , et al., 2002). Figure 14.

4.2 Quantification of PHA

PHA was estimated by the spectroscopy at 235nm and reported as standard deviations. After the PHA crystals was collected, weighted then convert into crotonic acid at 100° C, each sample was diluted 10^{-1} to 10^{-4} and OD was taken with Sulfuric acid as blank set. Figure 16

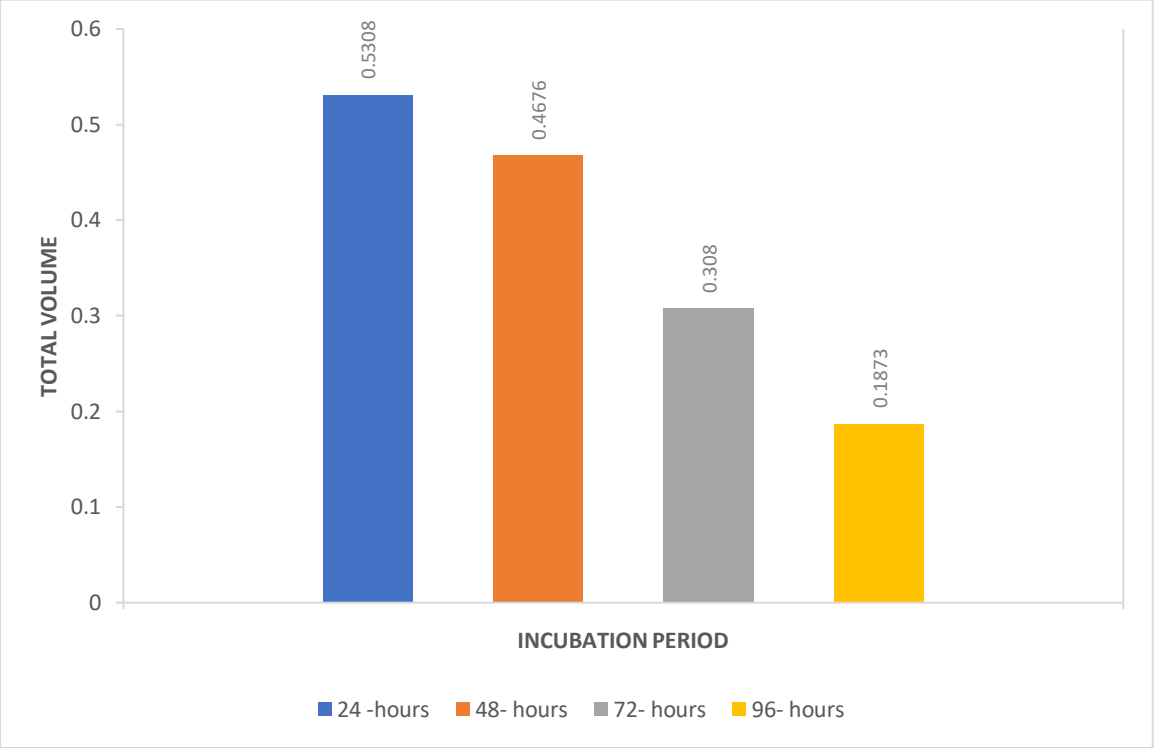

Figure 15 Quantification results of PHA

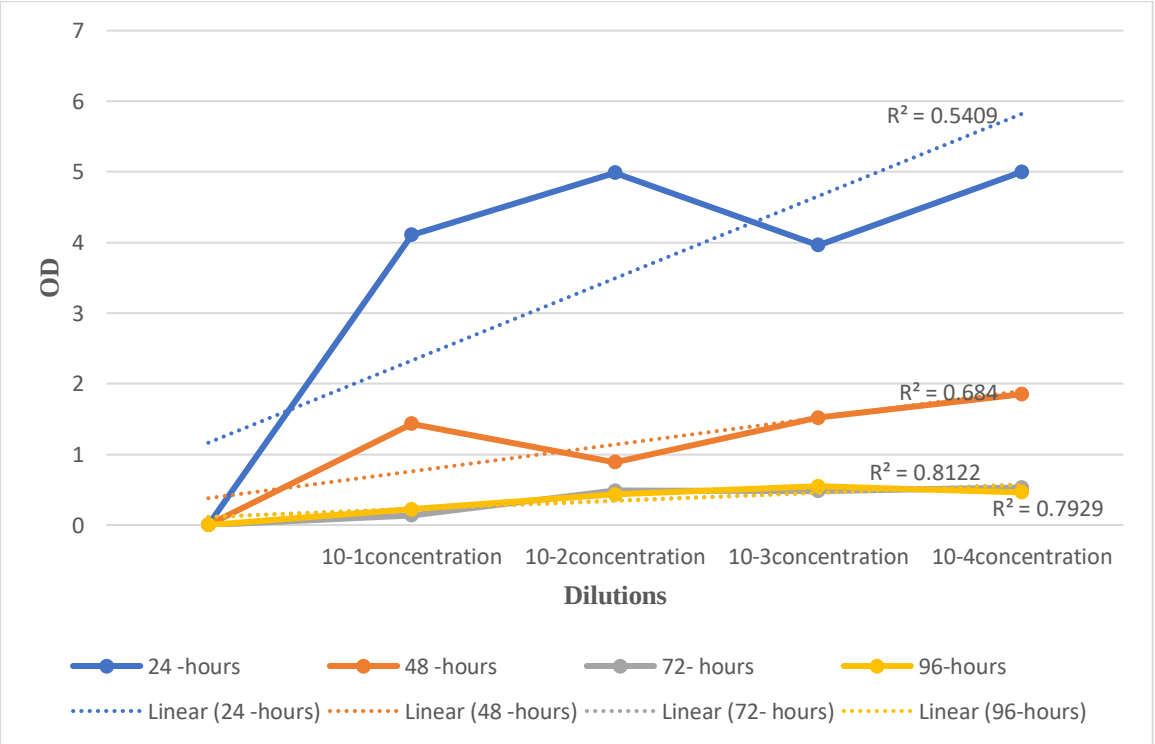

Figure 16 PHA Production profiles

CHAPTER 5
Conclusions

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Polyhydroxyalkanoates (PHA) crystal was successfully produced from food waste using *R. eutropha* under specific condition. The method of PHA extraction also influences the quality of polymer. PHA from *R. eutropha* can be alternative and possible solution to dumping waste and dumping food waste.

The objective of this work was to produce PHA by using low C-source i.e., food waste, which is also a waste and creating pollution and several types of disease. Reducing food waste and producing biopolymer from the food waste is also major objective of this work and producing alternative of synthetic polymer with the help of *R. eutropha.* In this study, bacteria sample were taken from microbiology lab of Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, for the purpose of production of biopolymer using food waste as a C-source. To obtain PHA from *Ralstonia Eutropha,* food waste used as C-source at different incubation period. All synthesized intracellular inclusion during growth on starch C-source. *Ralstonia Eutropha* cultivation were proven promising for PHA production. The inclusions were predominantly identified as Polyhydroxyalkanoates using and spectrophotometer and plot graph as suggested by Slepecky & Law.

In this work, for the conformation of PHA producing bacteria Nile blue A was used. Nile Blue A is a water-soluble basis oxazine dye, which has higher specificity than Sudan Black B for PHA screening and glow a bright-orange fluorescence on exposure to UV. After conformation of PHA producing bacteria, limiting other sources and provide only food waste as C-source in 250 ml of distilled water with food waste powder in 4 different Erlenmeyer flask of 500ml and incubated at 24, 48, 72, 96 hours. Better growth found in 24 hours of incubation as shown in figure 10, 11, 12, and 13. And after taking Optical Density (OD) of all the sample of crystal PHA 24 hours of incubated sample's crystal found good as compared to other.

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