Methane Production from Poly(lactic Acid) (PLA) and Polyhydroxybutyrate (PHB) Biodegradable Plastics with Anaerobic Granulated Sludge

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Abstract: - In this study, using *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria under mesophilic (38 ± 1ºC) and thermophilic (58 ± 1ºC) conditions in anaerobic granulated sludge taken from Pakmaya Yeast Factory in Izmir, Turkey; Methane production from biodegradable plastics with poly(lactic acid) (PLA) and polyhydroxybutyrate (PHB) was investigated. Effect of different operating parameters, increasing biodegradation times (from 10 days to 500 days), different inoculum-substrate ratios (ISRs) (16, 8, 4, 2, 1) and increasing biochemical methane potential (BMP) times (between 10 day and 500 days) for the production of methane gas from PLA and PHB biodegradable plastics in anaerobic granular sludge waste; *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria were operated during the anaerobic digestion process under anaerobic conditions at mesophilic (38 ± 1ºC) and thermophilic (58 ± 1ºC) experimental temperatures. PLA biodegradable plastics were operated at optimum pH=7.6. PHB biodegradable plastics were carried out at optimum pH=8.1. Predicting the biodegradation behavior of PLA and PHB biodegradable plastics with BMP tests; It is found that the ISR parameter plays a very important role. This study showed that temperature plays a key role in the aging of microorganisms (*Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) during anaerobic digestion, the degradation of bioplastic materials (PLA and PHB) and the degradation of their natural properties. The increase in temperature from mesophilic conditions to thermophilic conditions increased the activities of methanogenic bacteria such as *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224. The maximum cumulative CH_4(g) production was measured at 630 NL CH_4 / kgVS for PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria), at ISR=16 value, after 100 days, at pH=8.1 and at 58±1ºC, respectively. The maximum 97% biodegradation efficiency was observed for PHB biodegradable plastics after 100 days, at pH=8.1 and at 58 ± 1ºC thermophilic conditions, respectively.

Key-Words: - Anaerobic granulated sludge; ANOVA statistical anaysis; Baker's yeast; Biodegradable plastics; Methane production; *Methanococcus vannielii* DSM 1224; *Methanosarcina barkeri* DSM 800**;** Polyhydroxybutyrate (PHB); Poly(lactic acid) (PLA); Volatile fatty acids (VFAs).

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1 Introduction

Some plastics obtained from petroleum products are not biodegradable and have a very long half-life; They can remain in the ecosystem for up to hundreds of years. This long period significantly affects ecological steady-state conditions; It can cause acute or chronic toxicity in all organisms such as humans, bacteria, algae, yeasts, fish and viruses. This cause to the disruption of aquatic and land ecosystems, [1], [2]. Generally, accumulated plastic waste is sent to landfill or incinerated. The majority of the wastes present in marine aquariums originated from disposable plastic and non-biodegradables, [3]. Although, advances were carried out in plastic industry, some drawbacks to the environment like their resistance to biodegradation is still. Procedures for breaking down plastic are not sufficient; For this reason,

some synthetic polymers disposed of in landfills or the marine environment persist in water and soil environments, [4].

In recent years, more environmentally friendly, degradable plastics have been produced in order to protect the environment. These plastics can be degraded through conventional treatment processes and recycled for reuse, [5]. Every year, 400 million tons of plastics are generated, and biological degradable plastics ratio was 1% of the whole generation of plastics, [5]. In recent years, the total production of biodegradable plastics has increased from 1.5 million tons to 2.6 million tons in 2022, and it is estimated that this production will increase by 4 times for 2028, [6].

There are three types of biodegradable plastics: A): Biodegradable and non-biodegradable plastics, exhibited properties like petrochemical counterparts such as bio-polyethylene and polyethylene (bio-PE and PE), bio-polyethylene terephthalate and polyethylene terephthalate (bio-PET and PET), bio-polypropylene and polypropylene (bio-PP and PP), [7], [8]. B) Petrochemical and biodegradable plastics such as polybutylene adipate terephthalate (PBAT), polybutylene succinate (PBS) and polycaprolactone (PCL); It represents 44.5% of the total bioplastic production capacity in 2019, [6]. C) Bio-based and biodegradable plastics such as starch blends, polylactic acid (PLA) and polyhydroxyalkanoate (PHA); It accounts for 19.6% of the total biodegradable plastic production rate in 2019, [6]. These plastics are more environmentally friendly compared to other biodegradable plastics mentioned above.

Management procedures for biodegraded plastics include: mechanical recycling, chemical recycling and organic recycling, [9]. Composting in industries is preferred for biodegradable plastics. Therefore, polymers are biodegradable according to the EN 13432 standard; and are described as biodegradable at least in industrial composting facilities, [6]. Measures taken into account; It can guide people about bioplastic waste and waste recycling rates. A good example of an environmental policy approach is the extension of liability for a product to the post-consumer phase of the product's life cycle, [6]. The biodegradable properties of some biodegradable plastics enable them to create valuable substrates; Other recycling biotechnologies based on the use of bioplastics, enzymes and microorganisms, such as composting and anaerobic digestion, enable reuse, [10], [11].

Methane production is the last step of anaerobic digestion, and is produced from acetic acid and hydrogen, which were formed by biodegradation of organics, [10]. Methanogenesis define the anaerobic metabolism ratio, and provide the substrate to convert methane gas [CH_4(g)] with high efficiency to ultimate step, [11]. Although, CH_4(g) and carbon dioxide gas [CO_2(g)] are produced during methanogenesis, low amounts of hydrogen sulfide, ammonia and water are also produced, [12]. 95% of biodegradable organic matter decomposes into gaseous products, the remaining 6% consists of biomass, [13].

Anaerobic digestion method: It provides excellent savings by reusing some of the energy released from CH_4(g) production, [6]. The changes of the operational conditions and variation the plastic types used during anaerobic biodegradation cause to a difficulty in the comparison of the data in different researchers. Anaerobic digestion occurs in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The organic substrate is degraded into biogas namely, CO_2(g) and CH_4(g) by different types of anaerobic bacteria and methanogenic Archaea. In energy saving, anaerobic treatment; It has been shown to be an important means of disposing of bioplastic bags containing biological waste and contaminated bioplastics. Under these conditions, carbon and nutrients can be reused, biogas can be produced as renewable energy, and non-biodegradable materials can be used as biofertilizers, [10]. Since bioplastics contained high carbon rings can be co-depredate with feedstocks containing optimum carbon to nitrogen ratios (C/N) such as agricultural residues, food wastes, manures and wastewater sludge, [6]. Based on these information's, the probable anaerobic biodegradability of bioplastics is important based on microbial diversity and environmental and operational conditions, [6]. However, all types of bioplastics cannot be managed by Anaerobic digestion effectively, [10]. The biodegradation time of biodegradable bioplastics is 4 to 7 times higher than the 40-day conventional hydraulic retention time of domestic biogas treatment plants, [10], [14], [15], [16], [17]. Under these conditions it is important to detect suitable ecofriendly and not treatments can be introduced to the anaerobic digestion of bioplastics for ultimate biodegradation.

Biodegradable plastics are synthesized from plant-based PLA. PLA accounted for 30% of biodegradable plastic production in 2019, [5]. Since the biodegradation efficiency of PLA is low for mesophilic anaerobic digestion, the degradation time is as long as 80 and 110 days, [18], [19]. PLA is a linear aliphatic polyester consist from renewable resources. It is synthetized by direct

polycondensation of lactic acid or by carbon polymerization of lactide, [20]. Lactic acid is produced by fermentation of various biomasses like corn, wheat, sugar cane, and sugar beet, [21]. As the hydroxyl group is asymmetric in benzene ring of lactic acid(L or D) PLA can, , have two different chemical structures depending on whether the chain of monomers is isotactic (L-PLA) or syndiotactic (D,L-PLA), [22]. The stereoisomeric L/D percentage of lactate components affect the crystallinity, thermal and mechanical properties of the produced PLA, [22], [23]. Therefore, L-PLA produced by polymerization of one isomer can crystallize, however, D,L-PLA produced from a mixture of isomers is amorphous, [24]. It can also be used as bioplastic for packaging and textile fibers.

Polyhydroxybutyrate (PHB), a biological thermoplastic polyester, can be produced from waste-derived carbon sources; It can be a biodegradable and environmentally friendly substrate for synthetic syntheses, [25], [26]. Recent studies have shown that some methanotrophic bacterial species can accumulate PHB through oxidation of CH_4(g), [27]. This can be used as an energy source and expand the scope of utilizing PHB, [28]. CH_4(g) is produced as the final product in the anaerobic digestion process in wastewater treatment plants. The use of methanotrophs to improve the produced CH_4(g) reduces the cost of production of this gas, [29].

Methanosarcina is the most commonly used methanogen in different anaerobic digestions, representing 97% of the total archaea used. Methane-producing organisms are defined as Archea and their genes are as follows: *Methanococcus, Methanosarcina, Methanobacterium, Methanothermobacter, Methanobrevibacter* and *Methanosaeta* [30]. Hydrogenotrophic methanogens such as *Methanoculleus* and *Methanothermobacter* are more dominant in thermophilic anaerobic digesters that metabolize different PLA-based biodegradable plastics [31]. *Methanothermobacter* dominates during thermophilic anaerobic digestion of PLA wastes [32]. In the study, *Methanoculleus* and *Methanosarcina* were the other most abundant methanogenic Archaea in thermophilic reactors metabolizing biodegraded plastics. Hydrogenotrophic methanogens from the *Methinobacteriaceae* family have been found as the dominant bacteria in thermophilic reactors metabolizing PLA [33], [34]. *Methanosarcina* metabolizes both hydrogenotrophic and acetoclastic metabolism and shows effective methane production [35]. Low pH and accumulation of doses of volatile fatty acids (VFA) in anaerobic reactors lead to increased numbers of *Methanosarcina* (acetoclastic and hydrogenotrophic methanogens) and *Methanothermobacter* (hydrogenotrophic methanogens). A low *Methanoculleus* ratio negatively affects VFA accumulation [36]. When comparing pure methanogen cultures, resistance to VFAs > 90 mg/l was observed in cultures of *Methanosarcina barkeri*, *Methanothermobacter marburgensis* and *Methanobacterium formicicum* [37]. Among the methanogens, the physiology and metabolism of *Methanococcus maripaludis* S2 differ from others in its high specific growth rate (μ), its genetic manipulability due to its growth and resistance at high temperatures (35–39°C), [38] and its effective biodegradability, [39] It is different. These advantages make *Methanococcus maripaludis* a good laboratory Archaea for physiological and biotechnological studies [40]. *Methanococcus maripaludis*, an autotrophic, hydrogenotrophic methanogen, can be used to convert CO_2(g) to CH_4(g) (CO_2-biomicroplastic), [41], [42], [43]. On the other hand, *Methanococcus maripaludis* was applicable to the wastewater treatment plants, [44]. With high biotechnological utilization of the Methanogenic Archaea such as, *Methanococcus maripaludis* in general, some toxicological effects of heavy metals such as cadmium, chromium, copper (Cu), mercury (Hg), uranium (U), zinc (Zn), and VFAs (acetate ($C_2H_3O_2^-$) and propionate ($C_3H_5O_2^-$)), is still limited, [41], [43], [45], [46], [47], [48], and of *Methanococcus maripaludis* in particular, [43], [49], [50].

In general, high biotechnological use of Methanogenic Archaea such as *Methanococcus maripaludis* and some toxicological effects of heavy metals such as cadmium, chromium, copper (Cu), mercury (Hg), uranium (U), zinc (Zn) and VFAs [acetate, $(C_2H_3O_2)^-$] and [propionate, $(C_3H_5O_2^-)$], removal is possible, [41], [43], [45], [46], [47], [48], but studies on this subject are still limited, especially for *Methanococcus maripaludis*, [43], [49], [50].

In this study, poly(lactic acid) (PLA) and polyhydroxybutyrate (PHB) were biologically synthesized using Methanosarcina barkeri DSM 800 and Methanococcus vannielii DSM 1224 methanogenic bacteria under mesophilic and thermophilic conditions in anaerobic granular sludge taken from Pakmaya Yeast Factory in Izmir, Turkey. Methane gas production from degradable plastics was investigated. The effects of some operational conditions like increasing biodegradation times (from 10 days to 500 days),

different ISRs (16, 8, 4, 2, 1), and increasing BMP times (between 10 days and 500 days) on the methane yields were investigated in both Archae for anaerobic digestion of PLA and PHB biodegradable plastics under anaerobic conditions at mesophilic (38 ± 1°C) and thermophilic (58 ± 1°C) experimental temperatures, respectively. PLA biodegradable plastics were operated at pH=7.6. PHB biodegradable plastics were operated at pH=8.1.

2 Materials and Methods

2.1 Microorganisms

2.1.1 *Methanosarcina barkeri* DSM 800

Cultures of the archaeal strain *Methanosarcina barkeri* DSM 800 were purchased from DSMZ (DSMZ, Braunschweig, Germany). *Methanosarcina barkeri* DSM 800 bacteria were cultured at 37°C and at pH=7.5 in a new stock culture according to literature, [51]. New stock cultures were maintained by monthly subculture from a frozen glycerol stock using a 10% v/v inoculum in DSM 120 medium (pH=6.8), [52]. DSM 120 medium: 0.35 g/l K_2HPO_4, 0.23 g/l KH_2PO_4, 0.5 g/l NH_4Cl, 0.5 g/l $MgSO_4.7H_2O$ (Epsomite or Epsom salt), 0.25 g/l $CaCl_2.2H_2O$, 2.25 g/l NaCl, 2 mg/l $FeSO_4.7H_2O$, 1 ml/l trace element solution, 2 g/l yeast extract and 2 g/l casitone agar (contents: 9 g/l casitone + 5 g/l yeast extract, 0.54 g/l KH_2PO_4, 3.34 g/l Na_3PO_4, 10 g/l $Na_3C_6H_5O_7$, 20 g/l $C_6H_{12}O_6$, 18 g/l agar and final pH=7.0 ± 0.2) was used. 50 ml of medium was added to 120 ml serum bottles and sparged with 100% nitrogen gas [N_2(g)] before being sealed with butyl rubber tops and autoclaved. Additional media components (CH_3OH, $C_3H_8ClNO_2S$, $Na_2S.9H_2O$ and $NaHCO_3$) were prepared in the same way, by flushing with 100% N_2(g) before being sealed and autoclaved. These were added to bottles of sterile DSM 120 medium before inoculation using an aseptic syringe method. All inoculations and sub-culturing were performed in an anaerobic chamber (PLAS-LAB Simplicity 888, PLAS-LABS, USA.). Methanosarcina *barkeri* DSM 800 cultures were incubated at 37°C and pH=7.0 for 4 days and growth was monitored by optical density measurements at 600 nm (OD_{600}). To maintain reproducibility of results and to avoid phenotypic drift from repetitive culturing, experiments were started from maintained stock cultures and were sub-cultured no more than 3 times.

2.1.2 *Methanococcus vannielii* DSM 1224

Cultures of the archaeal strain *Methanococcus vannielii* DSM 1224 were purchased from DSMZ (DSMZ, Braunschweig, Germany). *Methanococcus vannielii* DSM 1224 bacteria were cultured at 37°C and at pH=7.5 in a formate/mineral salts medium, [53]. The composition of stock solution for media: General salt solution contains to 0.67 g/l KCl, 5.50 g/l $MgCl_2.6H_2O$, 6.90 g/l $MgSO_4.7H_2O$, 1.00 g/l NH_4Cl, 0.28 g/l $CaCl_2.2H_2O$. Trace mineral solution contains to 1.5 g/l $C_6H_9NO_6$, 0.1 g/l $MnSO_4.H_2O$, 0.2 g/l $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.1 g/l $CoCl_2.5H_2O$, 0.1 g/l $ZnSO_4.7H_2O$, 0.01 g/l $CuSO_4.5H_2O$, 0.025 g/l $NiCl_2.6H_2O$, 0.2 g/l Na_2SeO_3, 0.1 g/l $Na_2MoO_4.2H_2O$, 0.1 g/l $Na_2WO_4.2H_2O$. Iron stock solution occurs to 2 g/l $Fe(NH_4)_2(SO_4)_2.6H_2O$, 100 µl/l concentrated HCl. Trace vitamin solution contains to 2 mg/l $C_{10}H_{16}N_2O_3S$, 2 mg/l $C_{19}H_{19}N_7O_6$, 10 mg/l $C_8H_{11}NO_3.HCl$, 5 mg/l $C_{12}H_{18}Cl_2N_4OS$, 5 mg/l $C_{17}H_{20}N_4O_6$, 5 mg/l C_5H_4NCOOH, 5 mg/l $C_{18}H_{32}CaN_2O_{10}$, 0.1 mg/l Vitamin B_{12}, 5 mg/l $C_7H_7NO_2$, 5 mg/l $C_8H_{14}O_2S_2$. Modification of basal medium for *Methanoccus vannielii* DSM 1224 includes general salt solutions, trace mineral solution, iron stock solution, trace vitamin solution, 293 g/l (18 ml/l) NaCl, and modification of basal medium for growth on formate (McF) occurs 300 ml/l deionized water, 80 ml/l HCOONa (5 M), 200 ml/l $C_4H_8N_2O$ (1 M, pH=7.0).

Preparation of 100 ml McF liquid medium: In a 500 ml round-bottom flask, the indicated components of *Methanococcus vannnielii* DSM 1224 growth format/mineral salts medium were mixed, the stopper was loosely closed and the solution was heated to N_2/CO_2 (80/20, v/v) was boiled under the flow for 5-10 seconds. The solution was allowed to cool under N_2/CO_2 flow at 35-70 kPa and 0.05 g of $C_3H_{10}ClNO_3S$ or $HSCH_2CH_2SO_3Na$ was added to reduce the medium. The vial was tightly capped and transferred to the anaerobic chamber. The solution was distributed into Balch tubes or serum bottles. The tubes were closed with 20 mm blue septum stoppers (Bellco Glass, Inc.), and each stopper was secured with an aluminium seal. Before inoculation of all media, 0.1 ml of 2.5% $Na_2S.9H_2O$ was added anaerobically to every 5 ml of media using anaerobic procedures. The tubes were removed from the chamber and degassed by a three-cycle H_2/CO_2 and vacuum procedure. Each tube was pressurized to 137 kPa H_2/CO_2 and sterilized by autoclaving the tubes at 121°C for 20 min. After inoculation, up to 275 kPa was pressurized into each tube. It was incubated overnight at 37°C and growth was monitored by optical density measurements at 540 nm (O_{540}). The pH of the culture was between 8.0 and 8.5.

2.2 Biodegradable Plastic Materials

Thin layer chromatography grade cellulose (positive control) powder with particle size less than 20 mm was purchased from Merck (Cellulose Microcrystalline, Merck, Darmstadt, Germany) and then milled using A plant chipper (GHE 355, Stihl®, Waiblingen, Germany) was purchased. PLA and PHB bioplastics, certified as biodegradable under industrial composting conditions according to the EN 13432 standard, were used for this experiment. PHB was purchased from (Sigma-Aldrich, Germany). Semi-crystalline PLA was obtained from NaturePlast® (NP SF 141). The PLA powders were prepared by mechanical crushing methods as previously described, [54], [55]. Both PLA and PHB bioplastics had an average particle size of 1.01 ± 0.51 mm determined by laser granulometry (Mastersizer 3000, Malvern, United Kingdom). For this purpose, it was ground using liquid nitrogen and a centrifugal mill (ZM 100, Retsch, Haan, Germany) and obtained in the form of pellets. PHB was separated by sieving after grinding with a mortar and pestle to obtain particles in this size range. The average molecular weight and polydispersity of PLA and PHB are 80,000 Mn, 1.72 Mw/Mn and 152,000 Mn, 2.28 Mw/Mn.

2.3 Anaerobic Granulated Sludge

The characterization values of anaerobic granulated sludge from Pakmaya baker's yeast producing factory in İzmir, Turkey was shown in Table 1.

* Table 1 can be found in the Appendix section.

2.4 Experimental Procedure

Two laboratory inoculums were used for the Biochemical Methane Potential (BMP) testing. Both of inoculums were prepared from anaerobic granulated sludge of Pakmaya baker's yeast producing factory in İzmir, Turkey, and acclimated to either mesophilic (38^{o}C) or thermophilic (58^{o}C) anaerobic conditions. The inoculums were kept under anaerobic digestion conditions for several months before being used in the BMP test and fed twice a week with a mixture of wastewater treatment plant sludge and green grass from the Pakmaya baker's yeast production factory in Izmir, Turkey. The ability of the laboratory inoculum to convert organic compounds to CH_4(g) was regularly verified by using the BMP test on cellulose. Other parameters such as the pH, oxidation reduction potential (ORP), ammonia (NH_3) titration, VFAs content, and alkalinity were measured regularly to ensure the quality of the inoculum. Before their use in BMP testing, the inoculums were sieved at < 2 mm in order to remove part of the non-degraded organic fraction and like this reduce the endogenous CH_4(g) production from the inoculums.

The total solids (TS) and the volatile solids (VS) contents of the various samples such as, biodegradable plastics, inoculums, and cellulose were determined by drying at 105^{o}C until constant weight and calcination at 550^{o}C for 4 h according to Standard Methods (2022), [56]. Elemental analyses, for example carbon (C), hydrogen (H_2), nitrogen (N_2), and sulfur (S_2) were performed on plastic and cellulose samples using an organic elemental analyzer (vario MACRO cube, Elementar, Langenselbold, Germany). The oxygen (O_2) content was estimated by the difference between the VS, S, C, H_2, and N_2 contents. The characteristics of the inoculum and plastics are shown in Table 2.

* Table 2 can be found in the Appendix section.

NH_3 titration was performed using a designated kit (Spectroquant® Ammonium Cell Test). The VFAs content was measured by a gas chromatography-mass spectrophotometry (GC-MS) (Agilent 8890N GC – Agilent 5989 inert MSD). The volatile organic acids (VOA) to total inorganic carbonate (buffer capacity) (TIC) ratios (VOA/TIC ratios) were performed by titration using sulfuric acid (0.1 N), [57].

2.5 Basics of Anaerobic Digestion

The anaerobic digestion process is the conversion of organic matter into biogas [mainly composed of CO_2(g) and CH_4(g)] and digestate [undegraded fraction, rich in Nutrients] in an oxygen-free (or oxygen-free) environment.

Anaerobic digesters are mainly operated at two temperature ranges, namely mesophilic (35-38°C) or thermophilic (55-58°C). There are three main full-scale reactor configurations. These are designed to treat feedstocks with different total solids (TS) contents. Up-flow anaerobic sludge blanket (UASB) or anaerobic fluidized bed technologies are used to treat liquid feedstocks (< 3% TS), especially from urban wastewater and agro-food sectors. Continuous stirred-tank reactors (CSTR) are used to process feedstocks with a TS content between 8 and 15%. Solid-state anaerobic digestion (SS-AD) are designed for feedstocks with TS contents higher than 15% and are classified as dry batch anaerobic digestion and dry plug-flow anaerobic digestion.

Anaerobic digestion of sludge is a complex biochemical process in which organic macromolecules are broken down into simpler compounds, usually CO_2(g) and CH_4(g), [58], [59].

Each step of digestion is mediated by special microorganisms that hydrolyze polymeric substances through enzymatic action. The resulting monomers are further metabolized to alcohols, short-chain fatty acids, $H_2(g)$ and $CO_2(g)$, [60], [61], [62]. Anaerobic digestion has long been used to process sludge, decreasing its total mass and improving dewaterability. It also provides interchangeability. Additionally, anaerobic digestion; It helps stabilize sludge by removing easily decomposed organic matter, reducing susceptibility to decay, providing partial sanitization and reducing offensive odors, [63]. Anaerobic digestion is also used for treating wastewater with high loads of readily biodegradable organic matter, [64], [65], [66]. Organic fractions of municipal, industrial and agricultural wastes through anaerobic digestion; They can be used for biotechnological purposes or in special agricultural biogas plants to grow biomass for energy production, [60], [61], [67].

Anaerobic digestion is a four-step process where each successive step requires specific conditions and process parameters, [68]. The steps are generally classified as: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The first two (hydrolysis and acidogenesis) are collectively referred to as acidogenic fermentation (after their end product), whereas acetogenesis and methanogenesis are known as methanogenic fermentation, as they lead directly to the production of $CH_4(g)$, [69]. The flow diagram showing the transformations of the raw material during fermentation is presented in Fig. 1.

* Fig. 1 can be found in the Appendix section.

2.6 Analytical Methods

All experimental parameters were measured according to the Standard Methods (2022), [56].

The total gas generations and the gas composition were measured in the start-up period while the methane generation was measured under steady state conditions in the digester. The total gas generation was detected by the water displacement method. The biogas composition was analyzed by a gas chromatography-mass spectrometer (GC-MS). A gas chromatograph (Agilent Technology Model 8890N GC) was equipped with a mass selective detector (Agilent Technology Model 5989 inert MSD). A 2 ml sample volume was applied by injecting into the GC-MS. Mass spectra were recorded using a VGTS 250 spectrometer equipped with a capillary SE 52 column (HP5-MS 30 m, 0.25 mm ID, 0.25 μm) at 220°C with an isothermal program for 10 min. The initial oven temperature was kept at 55°C for 1 min during 2 min, then the time was increased to 5.5 min. Helium (He) was used as the carrier gas at constant flow mode (1.7 ml/min, 49 cm/s linear velocity). The calibration was carried out with a standard gas composed of 29% $CO_2(g)$, 3% $O_2(g)$, 8% $N_2(g)$ and 60% $CH_4(g)$, respectively.

VFA concentrations were measured after centrifugation of samples at 14000 rpm for 40 min. in a GC-MS (Agilent 8890N GC – Agilent 5989 inert MSD). As carrier gas $N_2(g)$ was used. All other pollutant assays were performed according to the Standard Methods (2022), [56].

2.7 Biochemical Methane Potential (BMP) Test

The BMP test is a procedure developed to determine the methane production of a given organic substrate during its anaerobic digestion at a lab scale. It is a reliable method to obtain the extent and rate of conversion of organic matter to methane. Pilot-scale experiments use more realistic conditions than BMP testing: feeding is continuous or semi-continuous and various parameters (biological, operational, performances) are monitored during the test. Pilot experiments provide precious insights regarding the process performance and stability over a long period of time. An important parameter that can influence anaerobic digestion performances is the C/N ratio of the feedstock. A carbon to nitrogen ratio was suggested for anaerobic digestion ranging from 20/1 to 30/1 for preventing both nutrient limitation and ammonia toxicity, [70]. Protein-rich wastes for example, food wastes or municipal sludges have C/N ratios ranging from 6/1 to 16/1, respectively. By contrast, most biodegradable plastics contain carbon but no nitrogen. Thus, co-digestion of biodegradable plastics with proteinaceous substrates can increase the C/N ratio to the suggested values and result in a more stable process, [19], [71], [72].

Biodegradable plastics (PLA and PHB) and cellulose (positive control) were tested in 500 ml batch bottles under mesophilic (38 ± 1°C) or thermophilic (58 ± 1°C) conditions. Although, PHB is readily biodegradable under mesophilic conditions, it has not been tested with the methanogenic bacteria *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 under thermophilic conditions. In the experimental protocol of BMP test, previous literature studies were used, [73], [74]. Each set of conditions was run in triplicate. The BMP bottles were filled with 300 ml of inoculum, water, and test material mixture, as described in Table 3.

* Table 3 can be found in the Appendix section.

While the amount of inoculum in various vials was the same, the amount of water and testing material content were respectively varied to have the same working volume in each vial and to test different inoculum-to-substrate ratios (ISRs). Five ISRs were tested: 16, 8, 4, 2, and 1. A triplicate of blank control, without test material, was also assayed. The positive control (i.e., cellulose) was tested with an ISR of 4. Before placing a cap equipped with a gas-tight connector on the bottles, the gas phase was flushed out with N_2(g) (Alphagaz™ with SMARTOP™, Air Liquide, France) to ensure anaerobic conditions. The daily biogas production was calculated from the pressure increase in the bottles measured by a manometer (2023P, Digital Instrumentation Ltd, Worthing, United Kingdom). The BMP bottles were shaken by hand once a day to mix the reactor volume. The biogas composition was determined once a week on the biogas accumulated in the headspace of the bottles. BMP bottles were plugged to a gas chromatograph (490-PRO Micro GC, Agilent Technologies, Inc., USA) equipped with two columns. The first column (M5SA 10 m, Agilent, USA) was used at 80°C and 200 kPa to separate O_2(g), N_2(g), and CH_4(g) using argon gas [Ar(g)] as the carrier phase; the second column (PPU 10 m BF) was used at 80°C and 150 kPa to separate the CO_2(g) from the other gases using helium gas [He(g)] as the carrier phase. The injector temperature was 110°C. Gaseous compounds were detected using a thermal conductivity detector. The calibration was carried out using two standard gases composed of 8% CO_2(g), 1% O_2(g), 81% N_2(g), 10% CH_4(g) and 30% CO_2(g), 5% O_2(g), 25% N_2(g), and 40% CH_4(g) (special gas, Air Liquide®, France). After the gas chromatography, the pressure of the headspace was equilibrated to atmospheric pressure. The biogas production of the blank control (without test material), which was endogenous to the inoculum, was subtracted from the production of the other bottles. All of the results are presented for normalized conditions of temperature and pressure (P_{atm}, 0°C) and corrected for moisture. The pH and redox were controlled at the beginning of the test to verify the initial state of the inoculum, and at the end of the test in order to observe potential acidification that would be inhibitory for the CH_4(g) production.

2.8 Biodegradability Tests

Biodegradation of the samples was calculated based on the theoretical CH_4(g) production (BMP_{th}) calculated from the elemental characterization ($C_xH_yO_zN_nS_s$) according to Eq. 1, [75], [76]:

$$BMPth\left[NL(CH_4).kg^{-1}\left(C_xH_yO_zN_nS_s\right)\right] = \frac{22.4\ \mathrm{x}\left(\frac{x}{2}+\frac{y}{8}-\frac{z}{4}-\frac{3n}{8}-\frac{s}{4}\right)}{12x+y+16z+14n+32s} \quad (1)$$

where, 22.4 is the molar volume of an ideal gas. The biodegradation was calculated by comparing the observed CH_4(g) production (BMP_{exp}) and the theoretical CH_4(g) production as shown in Eq. 2:

$$Biodegradation\ (\%) = \frac{BMP_{exp}}{BMP_{th}}\ x\ 100 \quad (2)$$

2.9 Modelling

The cumulative CH_4(g) production curves of the various substrates were modeled according to a modified Monod-Gompertz model, [77], as shown in Eq. (3).

$$G(t) = G(0) * \exp\left[-\exp\left(\frac{R_{max}*exp(1)}{G(0)} * (\lambda - t) + 1\right)\right] \quad (3)$$

where; G(t): is the cumulative CH_4(g) production at time t in NL $CH_4.kg^{-1}$ VS, G(0): is the ultimate CH_4(g) produced in NL $CH_4.kg^{-1}$ VS, λ is the time lag in days, R_{max} is the CH_4(g) production rate in NL $CH_4.kg^{-1}$ VS $days^{-1}$.

The parameters of the model were determined for each condition using R software (version 3.6.2) and the Nonlinear Least Squares method (nlsLM function of the minpack.lm package).

2.10 Flux Uncertainties and Limits of Detection (LOD)

The measured flux includes the true flux (F) plus random (∈) and systematic (δ) error components for measurement system (x) at time (t) in Eq. (4):

$$F_{t,x} = F_t + \in_{t,x} + \delta_{t,x} \quad (4)$$

Systematic error can result from (I) incorrect calibration of instrumentation, (II) incomplete sampling of turbulent fluctuations, (III) failure to observe non-turbulent flows during weak mixing conditions, and (IV) potential underestimation of the flow energy used during mixing in the anaerobic digestion process.

The calculations were used to identify the main biodegradable plastics and calculate their biodegradation behavior in various anaerobic

digestion processes according to ISO 15985 (simulating high solid and thermophilic anaerobic digestion) and ISO 14853 (simulating semiliquid and mesophilic anaerobic digestion), [78]. While spectral corrections induce uncertainties of their own, we nevertheless assume here that after spectral corrections, remaining $\epsilon_{t,x} >> \delta_{t,x}$.

Before performing experimental error analysis; High-frequency CH_4(g) concentrations were remeasured separately from GC-MS measurements with a low-power open path analyzer (LI-7700, LI-COR Biosciences Inc.) and a closed path tracer gas analyzer (TGA100A, Campbell Scientific). Laser spectroscopy was used in both analyses.

2.11 Performed Statistical Data

ANOVA test was applied to the experimental data to determine F and P values and to show the significance between dependent and independent variables, [79]. Variation of the experimental data mean and standard deviation values was indicated by F ratio. F explain the variation of the data averages/expected variation of the date averages. P indicates the significance data, and d.f shows the freedom degrees. Regression analysis was applied to the experimental data to detect the regression coefficient R^2, [80]. These data were calculated using Microsoft Excel Program. Statistical comparison of the parameters was performed on R using ANOVA and Tukey's HSD at a probability of significance level $P \leq 0.05$.

All experiments were carried out three times and the results are given as the means of triplicate samplings. The data relevant to the individual pollutant parameters are given as the mean with standard deviation (SD) values.

3 Results and Discussions

3.1 Results of BMP Tests

Cumulative methane production during the anaerobic digestion process, PLA and PHB biodegradable plastics in anaerobic granular sludge with the inoculation culture consisting of a mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannnielii* DSM 1224 methanogenic bacteria; It was measured at various ISR values (16, 8, 4, 2 and 1), examined under mesophilic (38±1°C) and thermophilic (58±1°C) conditions. The properties of the inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) and PLA, PHB biodegradable plastics and cellulose (as a positive control or blank sample) were illustrated in Table 3.

The cumulative CH_4(g) productions were measured from the anaerobic digestion process of PLA and PHB at the various ISRs values (16, 8, 4, 2, and 1) under mesophilic (38±1°C) conditions for PLA (Fig 2a) and PHB (Fig 2b) biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) (Fig 2).

* Fig. 2 can be found in the Appendix section.

The cumulative CH_4(g) productions were found from the anaerobic digestion process of PLA and PHB at the various ISRs values (16, 8, 4, 2, and 1) under thermophilic (58±1°C) conditions for PLA (Fig 3a) and PHB (Fig 3b) biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) (Fig 3).

* Fig. 3 can be found in the Appendix section.

3.2 Results of Biodegradability Test under Anaerobic Conditions

The biodegradation yields were measured under both mesophilic (38±1°C) and thermophilic (58±1°C) anaerobic digestion situations for the PLA and PHB biodegradable plastics and cellulose (as a positive control or blank sample) was shown in Fig. 4.

* Fig. 4 can be found in the Appendix section.

The average biodegradation efficiencies based on theoretical methane production were obtained from PLA biodegradable plastics at pH=7.6 and PHB biodegradable plastics at pH=8.1 under mesophilic (at 38 ± 1°C) conditions after 500 days (Fig. 4a) and thermophilic (at 58 ± 1°C) conditions after 100 days (Fig. 4b), respectively.

2%, 4%, 8%, 10%, 12.5%, 14%, 16%, 18%, 20%, 23%, 38%, 49%, 57%, 65%, 71%, 74%, 79% and 84% biodegradation yields were measured after 10 days, 20 days, 30 days, 40 days, 50 days, 60 days, 70 days, 80 days, 90 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 400 days, 450 days and 500 days, respectively, for PLA biodegradable plastics, at pH=7.6, and at 38 ± 1°C mesophilic conditions (Fig 4a). The maximum 84% biodegradation efficiency was observed for PLA

biodegradable plastics after 500 days, at pH=7.6 and at 38 ± 1°C mesophilic conditions, respectively (Fig. 4a).

62%, 68%, 70%, 75%, 78%, 79%, 80%, 81%, 83%, 86%, 86%, 86%, 86%, 86%, 86%, 86%, 86%, 86% biodegradation efficiencies were measured after 10 days, 20 days, 30 days, 40 days, 50 days, 60 days, 70 days, 80 days, 90 days, 100 days, 150 days, 200 days, 250 days, 300 days, 350 days, 400 days, 450 days and 500 days, respectively, for PHB biodegradable plastics, at pH=8.1, and at 38 ± 1°C mesophilic conditions (Fig 4a). The maximum 86% biodegradation efficiency was observed for PHB biodegradable plastics after 500 days, at pH=8.1 and at 38 ± 1°C mesophilic conditions, respectively (Fig. 4a).

21%, 48%, 60%, 69%, 75%, 78%, 79%, 81%, 87% and 92% biodegradation yields were found after 10 days, 20 days, 30 days, 40 days, 50 days, 60 days, 70 days, 80 days, 90 days and 100 days, respectively, for PLA biodegradable plastics after 100 days, at pH=7.6 and at 58 ± 1°C thermophilic conditions, respectively (Fig. 4b). The maximum 92% biodegradation efficiency was obtained for PLA biodegradable plastics after 100 days, at pH=7.6 and at 58 ± 1°C thermophilic conditions, respectively (Fig. 4b).

13%, 14%, 25%, 74%, 76%, 79%, 84%, 88%, 93% and 97% biodegradation efficiencies were measured after 10 days, 20 days, 30 days, 40 days, 50 days, 60 days, 70 days, 80 days, 90 days and 100 days, respectively, for PHB biodegradable plastics after 100 days, at pH=8.1 and at 58 ± 1°C thermophilic conditions, respectively (Fig. 4b). The maximum 97% biodegradation efficiency was observed for PHB biodegradable plastics after 100 days, at pH=8.1 and at 58 ± 1°C thermophilic conditions, respectively (Fig. 4b).

The biodegradation yields were calculated by comparing the obtained methane production with the theoretical methane production (Table 3 and Eq. 1) and in calculating biodegradation efficiencies were used in Eq. 2. Methane production curves were then modelized using the Monod-Gompertz model (Eq. 3), which allows description of the data according to three parameters: the duration of λ is the lag phase (days), G0 is the ultimate CH_4(g) yield (NL CH_4 / kg VS), and R_{max} is the CH_4(g) production rate (NL CH_4 / kg VS.d), respectively. The calculated values of the model's parameters are listed in Table 4.

* Table 4 can be found in the Appendix section.

The Monod-Gompertz model was not suitable for all experimental situations. In some circumstances, the biodegradation efficiency was very close to ground zero point and for this reason, the Monod-Gompertz model calculated and predicted abnormal parameter values. For all other experimental conditions, very good correlation numbers such as $R^2 \geq 0.992$ were measured. These high correlation coefficients proved that the Monod-Gompertz model accurately describes the cumulative CH_4(g) production in experimental samples.

3.3 Results for PLA Biodegradable Plastics at Mesophilic Conditions

The different ISR values (16, 8, 4, 2 and 1) were operated with anaerobic digestion process under mesophilic conditions for PLA biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) after 500 days, at optimum pH=7.6, and at 38±1°C, respectively (Fig. 2a). The cumulative CH_4(g) productions were observed after the anaerobic digestion process for PLA biodegradable plastics after 500 days, at pH=7.6, and at 38±1°C mesophilic conditions, respectively (Fig. 2a).

At Fig. 2a, the cumulative CH_4(g) production values from PLA biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of Methanosarcina barkeri DSM 800 and Methanococcus vannielii DSM 1224 methanogenic bacteria) were ranged between 150 and 430 NL CH_4 / kgVS for ISR=16 value, between 80 and 370 NL CH_4 / kgVS for ISR=8 value, between 100 and 390 NL CH_4 / kgVS for ISR=4 value, between 100 and 395 NL CH_4 / kgVS for ISR=2 value, and between 90 and 350 NL CH_4 / kgVS for ISR=1 value, respectively, after 500 days, at pH=7.6, and at 38±1°C. The maximum cumulative CH_4(g) production from PLA biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of Methanosarcina barkeri DSM 800 and Methanococcus vannielii DSM 1224 methanogenic bacteria) was obtained at 430 NL CH_4 / kgVS, at ISR=16 value, after 500 days, at pH=7.6, and at 38±1°C, respectively (Fig. 2a).

PLA has been one of the most investigated biodegradable plastics to date. According to rigid pieces of PLA (3 mm and < 1 cm^2) did not biodegrade in 60 and 90 days, respectively, [18], [81]. Similar observations have been reported for smaller pieces of PLA. Such as, 0.15 mm particles

and 20 x 40 mm film did not exhibit any significant biodegradation in 40 and 100 days, respectively, [19], [82]. Therefore, methane production during the digestion of PLA at 35 ± 2°C were reported, [83], [84], [85], [86], [87]. Minor biodegradation levels were obtained with between 10% and 23% of the PLA being converted into methane in 20 to 40 days, [83], [85], [87]. An anaerobic digestion of PLA ground to 125-250 µm were examined over a long period of time (277 days), [86]. At the end of the test, the PLA was biodegraded between 29 and 49% (depending on the run) but the methane production did not reach a plateau as methane production was still increasing. They explained the low biodegradation rate of PLA by the fact that the bacteria present in the mesophilic digesters did not have the ability to biodegrade higher molecular weight PLA. The microorganisms were only able to use PLA after a reduction of its molecular weight caused by a random hydrolytic chain scission of the ester linkages, [88]. Also, a mesophilic anaerobic digestion of PLA was performed over a long period of time (280 days), [89]. After 40 days of lag phase, there were two phases of constant biogas production. Firstly, 1.4 L CH_4 / kg VS.d of biogas was produced between the 40th and 90th day, and then, between the 90th and 280th day, the biogas production increased to 2.6 L CH_4 / kg VS.d. Finally, after 280 days, the biogas production reached 66% of the theoretical value, although the plateau phase was nonetheless not reached, [89].

3.4 Results for PHB Biodegradable Plastics at Mesophilic Conditions

The different ISR values (16, 8, 4, 2 and 1) were examined with anaerobic digestion process under mesophilic conditions for PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) after 500 days, at optimum pH=8.1, and at 38±1°C (Fig. 2b). The cumulative CH_4(g) productions were measured after the anaerobic digestion process for PHB biodegradable plastics after 500 days, at pH=8.1, and at 38±1°C mesophilic conditions (Fig. 2b).

At Fig. 2b, the cumulative CH_4(g) production values from PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) were measured between 50 and 550 NL CH_4 / kgVS for ISR=16 value, between 60 and 510 NL CH_4 / kgVS for ISR=8 value, between 40 and 530 NL CH_4 / kgVS for ISR=4 value, between 50 and 470 NL CH_4 / kgVS for ISR=2 value, and between 30 and 80 NL CH_4 / kgVS for ISR=1 value, respectively, after 500 days, at pH=8.1, and at 38±1°C. The maximum cumulative CH_4(g) production from PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) was obtained at 550 NL CH_4 / kgVS, at ISR=16 value, after 500 days, at pH=8.1, and at 38±1°C, respectively (Fig. 2b).

Poly(3-hydroxybutyrate) (PHB) is the most widespread member of the polyhydroxyalkanoates family. Complete or near-complete biodegradation of PHB samples in a short time period have been reported for mesophilic digesters. A 19 mm film made of PHB Biopol® BX G08 (ICI, United Kingdom) was fully converted into CH_4(g) after only 9 days of incubation in various microbial inoculum, [90]. PHB is a very promising polymer given its ability to be biodegraded in non-harsh environments such as mesophilic anaerobic digestion, home composting, soil, etc., [14]. The short time needed to fully biodegrade PHB makes it compatible with the conventional hydraulic retention time used in industrial anaerobic digestion plants, [14]. Therefore, the CH_4(g) conversion differed significantly depending on the grade of PHB used. The near complete biodegradation of two PHB grades were found, [19]. Mirel M2100 (Metabolix) and methane-derived PHB from Mango Materials, while only 50 to 59% of ENMAT Y3000 (TianAn) and Mirel F1006 (Metabolix) were degraded at the same time. Poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) is a co-polymer of poly(3-hydroxyvalerate) (PHV) and poly(3-hydroxybutyrate), and it is also one of the main members of the PHA family. Similar to PHB, PHBV exhibited a very good level of biodegradation in a short time, [82], [83], [91], [92], [93], [94]. For example, a biodegradation level of 95% was reported in 30 days for PHBV powder (420 µm) with 8.4% hydroxyvalerate (HV), [92]. A lower conversion into CH_4(g) was reported between 29 and 55% in 40 and 42 days, respectively, [83], [90], [95].

3.5 Results for PLA Biodegradable Plastics under Thermophilic Conditions

The different ISR values (16, 8, 4, 2 and 1) were operated with anaerobic digestion process under thermophilic conditions for PLA biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina*

barkeri DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) after 100 days, at optimum pH=7.6, and at 58±1ºC, respectively (Fig. 3a). The cumulative CH_4(g) productions were found after the anaerobic digestion process for PLA biodegradable plastics after 100 days, at pH=7.6, and at 58±1ºC thermophilic situations, respectively (Fig. 3a).

The cumulative CH_4(g) production values from PLA biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) were observed to between 140 and 510 NL CH_4 / kgVS for ISR=16 value, between 120 and 460 NL CH_4 / kgVS for ISR=8 value, between 100 and 410 NL CH_4 / kgVS for ISR=4 value, between 90 and 440 NL CH_4 / kgVS for ISR=2 value, and between 80 and 400 NL CH_4 / kgVS for ISR=1 value, respectively, after 100 days, at pH=7.6, and at 58±1ºC, respectively (Fig 3a). The maximum cumulative CH_4(g) production from PLA biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) was measured at 510 NL CH_4 / kgVS at ISR=16 value, after 100 days, at pH=7.6, and at 58±1ºC, respectively (Fig. 3a).

The CH_4(g) conversion of PLA under thermophilic conditions was more effective than under mesophilic conditions. A high level of biodegradation of 82% to 90% was reported in a mean digestion time of 90 days, [14], [96], [97], [98], [99]. Other authors have found a lower level of biodegradation, between 40 and 60%, with a similar timeframe for the digestion, [18], [81], [84], [100]. However, it should be noted that the biodegradation levels were presented to 60% and 40%, respectively, were not the final biodegradation levels, as the BMP tests were stopped before they reached the plateau of methane production, [81], [84]. A decrease in the molecular weight of PLA was had a positive effect on the biodegradation kinetics, [98]. Thermophilic degradation of rigid pieces of PLA (1 x 1, 2 x 2, and 3 x 3 cm) assessed and observed negligible CH_4(g) production of approximately 10 to 30 L CH_4 / kg VS, [15].

3.6 Results for PHB Biodegradable Plastics under Thermophilic Conditions

The different ISR values (16, 8, 4, 2 and 1) were operated with anaerobic digestion process under thermophilic conditions for PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) after 100 days, at optimum pH=8.1, and at 58±1ºC, respectively (Fig. 3b). The cumulative CH_4(g) productions were observed after the anaerobic digestion process for PHB biodegradable plastics after 100 days, at pH=8.1, and at 58±1ºC thermophilic conditions, respectively (Fig. 3b).

At Fig. 3b, the cumulative CH_4(g) production values from PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) were obtained between 130 and 630 NL CH_4 / kgVS for ISR=16 value, between 150 and 600 NL CH_4 / kgVS for ISR=8 value, between 60 and 550 NL CH_4 / kgVS for ISR=4 value, between 95 and 515 NL CH_4 / kgVS for ISR=2 value, and between 80 and 130 NL CH_4 / kgVS for ISR=1 value, respectively, after 100 days, at pH=8.1, and at 58±1ºC, respectively. The maximum cumulative CH_4(g) production from PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) was measured at 630 NL CH_4 / kgVS, at ISR=16 value, after 100 days, at pH=8.1 and at 58±1ºC, respectively (Fig. 3b).

The digestion of PHB in thermophilic digesters (around 55°C) was found a very high level of biodegradation (between 73% and 88%) in a short time (between 18 and 20 days), [84], [97]. Also, noted near-complete mineralization of PHB but, strangely, was reported a very long digestion period (127 days), [101].

3.7 Artificial Neural Networks (ANN) Sample Approach

Artificial neural networks (ANNs) are increasingly used for gap-filling (CH_4) flow time series, [102], [103], [104]. The most important advantages of ANNs are (i) their capacity to model data with variable temporal periodicity and (ii) their independence from any prior assumptions regarding the functional relationship between independent and dependent variables, [105], [106].

In this ANN approach established routines were followed; A feedforward network with varying architectural complexity and tan-sigmoid transfer functions was used (Table 5).

* Table 5 can be found in the Appendix section.

Before network training, the 30-min streaming time series was evenly subsampled into training, validation, and testing subsets. Test subsets were withheld from initialization and validation of individual network trainings and were used only to tease out uncertainty in the final selected networks. Network training and validation were repeated multiple times, with increasing complexity (increasing the number of hidden layers and neurons per hidden layer).

Training variables tested included active sludge temperature (10 cm), anaerobic digestion temperature, active sludge heat flux (average of 5 heat flux plates at 8 cm depth), ambient active radiation (PAR), water table location, active sludge humidity and atmospheric pressure. The existence of water and steam deficit in anaerobic digestion was tested and observed. First, the ranked these variables according to their correlations with observed methane fluxes. These were then added to the training dataset step by step.

After training and validation of each neural network, the mean square error (MSE) and coefficient of determination of the modeled data were calculated by comparing with the stored test data. Then, among the data found, we selected the network with the least number of training variables, the lowest number of (hidden) layers and nodes, the lowest MSE and the highest R^2. The ANN routine, including random subsampling, training, and validation, was repeated n = 50 times to calculate the ANN-derived ensemble distribution of space-filled time series. The uncertainty of the ANN approach; It was then evaluated based on the ensemble range, and the resulting ensemble mean was used to fill the gap.

Artificial Neural Networks (ANN) Sample Approach and error analysis results are comparatively summarized in Table 5.

4 Conclusions

The average biodegradation efficiencies based on theoretical CH_4(g) production were obtained from PLA biodegradable plastics at pH=7.6 and PHB biodegradable plastics at pH=8.1 under mesophilic (at 38 ± 1ºC) conditions after 500 days and thermophilic (at 58 ± 1ºC) conditions after 100 days, respectively.

The maximum 84% biodegradation efficiency was observed for PLA biodegradable plastics after 500 days, at pH=7.6 and at 38 ± 1ºC mesophilic conditions, respectively. The maximum 86% biodegradation efficiency was measured for PHB biodegradable plastics after 500 days, at pH=8.1 and at 38 ± 1ºC mesophilic conditions, respectively.

The maximum 92% biodegradation efficiency was obtained for PLA biodegradable plastics after 100 days, at pH=7.6 and at 58 ± 1ºC thermophilic conditions, respectively. The maximum 97% biodegradation efficiency was observed for PHB biodegradable plastics after 100 days, at pH=8.1 and at 58 ± 1ºC thermophilic conditions, respectively.

The cumulative CH_4(g) productions were measured from the anaerobic digestion process of PLA and PHB at the various ISRs values (16, 8, 4, 2, and 1) were examined under mesophilic (38±1ºC) and thermophilic (58±1ºC) conditions for PLA and PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria). The properties of the inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) and PLA, PHB biodegradable plastics and cellulose (as a positive control or blank sample) were illustrated.

The maximum cumulative CH_4(g) production was obtained at 430 NL CH_4 / kgVS for PLA biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) at ISR=16 value, after 500 days, at pH=7.6, and at 38±1ºC, respectively.

The maximum cumulative CH_4(g) production was obtained at 550 NL CH_4 / kgVS for PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria), at ISR=16 value, after 500 days, at pH=8.1, and at 38±1ºC, respectively.

The maximum cumulative CH_4(g) production was measured at 510 NL CH_4 / kgVS for PLA biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) at ISR=16 value, after 100 days, at pH=7.6, and at 58±1ºC, respectively.

The maximum cumulative CH_4(g) production was measured at 630 NL CH_4 / kgVS for PHB biodegradable plastics in anaerobic granulated sludge with inoculum culture (the mixture of *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic

bacteria), at ISR=16 value, after 100 days, at pH=8.1 and at 58±1°C, respectively.

Predicting the biodegradation behavior of PLA and PHB biodegradable plastics with BMP tests; The ISR parameter was found to play a very important role. Except for the final, methane-blocking case, methane production varied significantly with the ISR rates used. An increase in methane production rate was noted in parallel with an increase in ISR. Based on these observations, it was realized that low ISRs (ISRs ≤ 2) should be avoided to avoid overloading.

This study showed that temperature plays a key role in the aging of microorganisms (*Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224 methanogenic bacteria) during anaerobic digestion, the degradation of bioplastic materials (PLA and PHB) and the degradation of their natural properties. The increase in temperature from mesophilic conditions to thermophilic conditions increased the activities of methanogenic bacteria such as *Methanosarcina barkeri* DSM 800 and *Methanococcus vannielii* DSM 1224.

Anaerobic digestion process is a very effective process in the production of CH_4(g) from anaerobic granular sludge and poly(lactic acid) (PLA) and polyhydroxybutyrate (PHB) biodegradable plastics. In the production of CH_4(g) from biodegradable plastics; It is very important to carefully check ISR values, pH values, temperature values, operating times and suitable conditions for the selected microorganisms.

In our future work, biodegradable PLA and PHB bioplastics; It is aimed to shed light on the better understanding of the life activities of microorganisms involved in the anaerobic digestion process, the development of biomagnification strategies and the removal of other biodegradable bioplastics at high rates under optimum conditions.

It is predicted that energy production efficiencies will increase further with the widespread use of ANN and space filling (CH_4) flow time series in the production of CH_4(g) from biodegradable bioplastics.

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APPENDIX

Table 1. The characterization values for the effluent of wastewater process from Pakmaya baker's yeast producing factory, İzmir, Turkey.

Parameters	**Unit**	**Values**
Flow rate	m^3/day	3150
Chemical oxygen demand (COD)	mg/l	6194 ± 1130
Chemical oxygen demand-dissolved (COD_{dis})	mg/l	4982
Total organic carbon (TOC)	mg/l	1065
Biochemical oxygen demand-5 days (BOD_5)	mg/l	3780 ± 740
Total Kjeldahl Nitrogen (TKN)	mg/l	274 ± 113
Ammonium-Nitrogen (NH_4-N)	mg/l	135 ± 15
Total phosphorus (Total-P)	mg/l	4 ± 3
Phosphate-phosphorus (PO_4-P)	mg/l	2.5
Sulfate ion (SO_4^{-2})	mg/l	484 ± 71
pH		6.45 ± 0.19
Total solids (TS)	mg/l	35550 ± 3662
Total suspended solids (TSS)	mg/l	587 ± 108
Total volatile suspended solids (TVSS)	mg/l	479 ± 102
Alkalinity	mg $CaCO_3$/l	1480 ± 183
Specific wastewater production as yeast cake	m^3/t yeast cake	167
Temperature	(°C)	20-28
Specific wastewater flowrate as molasses	m^3/t molasses	10
Color		Dark brown
Turbidity	NTU	2074
Conductivity	mS/cm	19.22

Table 2. The properties of biodegradable plastics, inoculum and cellulose (mean values ± standard deviation).

Parameters	**Unit**	**Mesophilic inoculum (38ºC±1ºC)**	**Thermophilic inoculum (58ºC±1ºC)**	**PLA**	**PHB**	**Cellulose**
pH	-	7.50 ± 0.10	7.61± 0.12	-	-	-
ORP	mV	- 355± 13	-380± 5	-	-	-
Ammonia (NH_3)	$gN\text{-}NH_3/l$	1.71± 0.10	1.80± 0.10	-	-	-
VOA/TIC	-	0.22± 0.02	0.30 ±0.02	-	-	-
VFAs	$g_{\text{eq acetate}}/l$	0.09± 0.01	0.15± 0.01	-	-	-
TS	(%) raw mass	3.70± 0.09	3.10 ±0.10	99.8± 0.01	99.6± 0.01	90.0± 0.001
TVSS	(%) raw mass	2.40± 0.07	2.14 ±0.06	99.5± 0.01	97.7± 0.01	99.8± 0.1
Carbon (C)	% TS	-	-	51.81± 0.20	53.27± 0.25	40.72± 0.10
$H_2(g)$	% TS	-	-	7.24± 0.02	7.05 ±0.03	6.51± 0.01
$N_2(g)$	% TS	-	-	0	0.25 ±0.02	0
Sulfur (g)	% TS			0	0.16 ±0.03	0.20± 0
$O_2(g)$	% TS			44.27	42.39	52.8
Theoretical $CH_4(g)$ potential	NL CH_4/kg VS			584	612	417

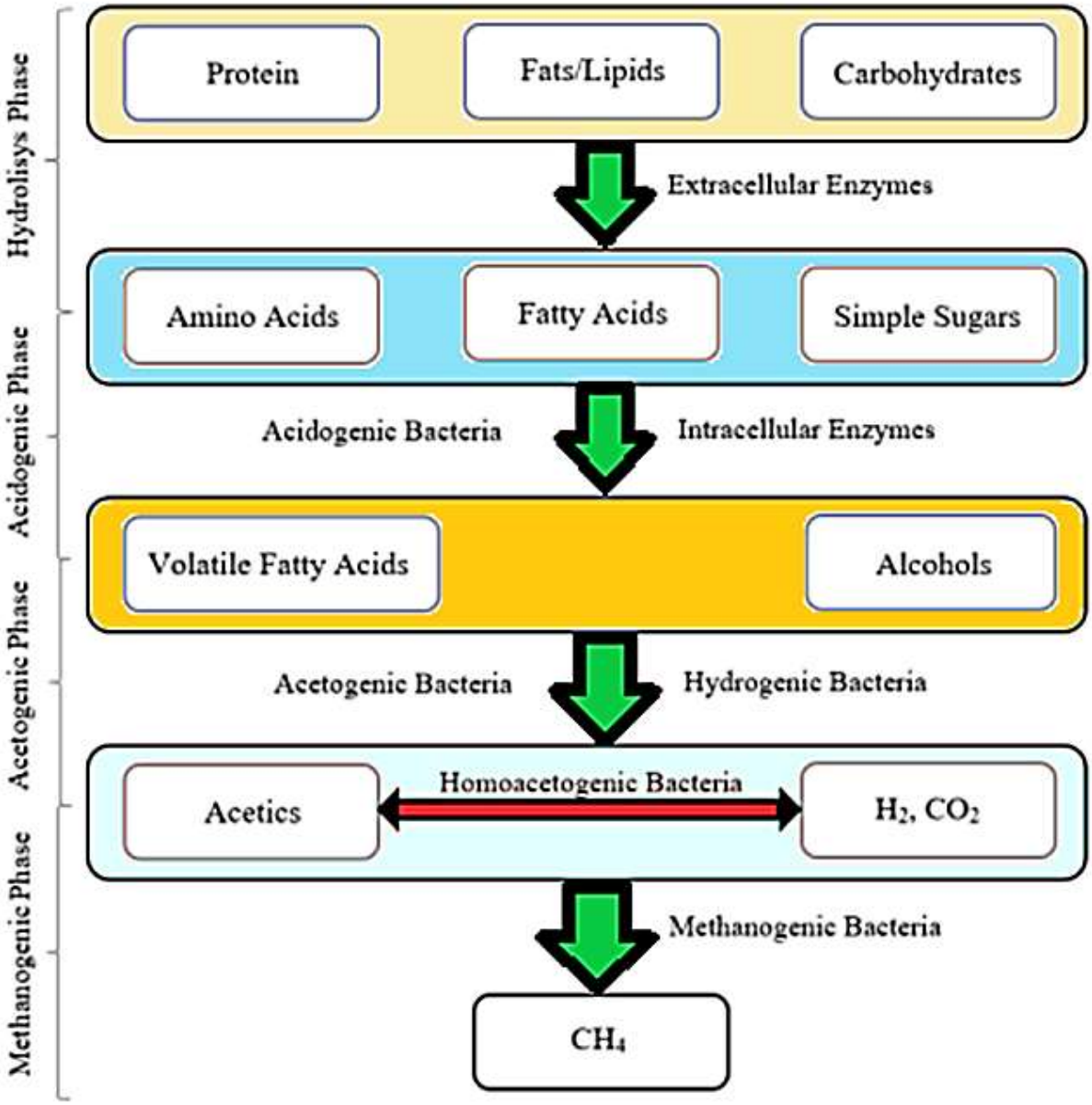
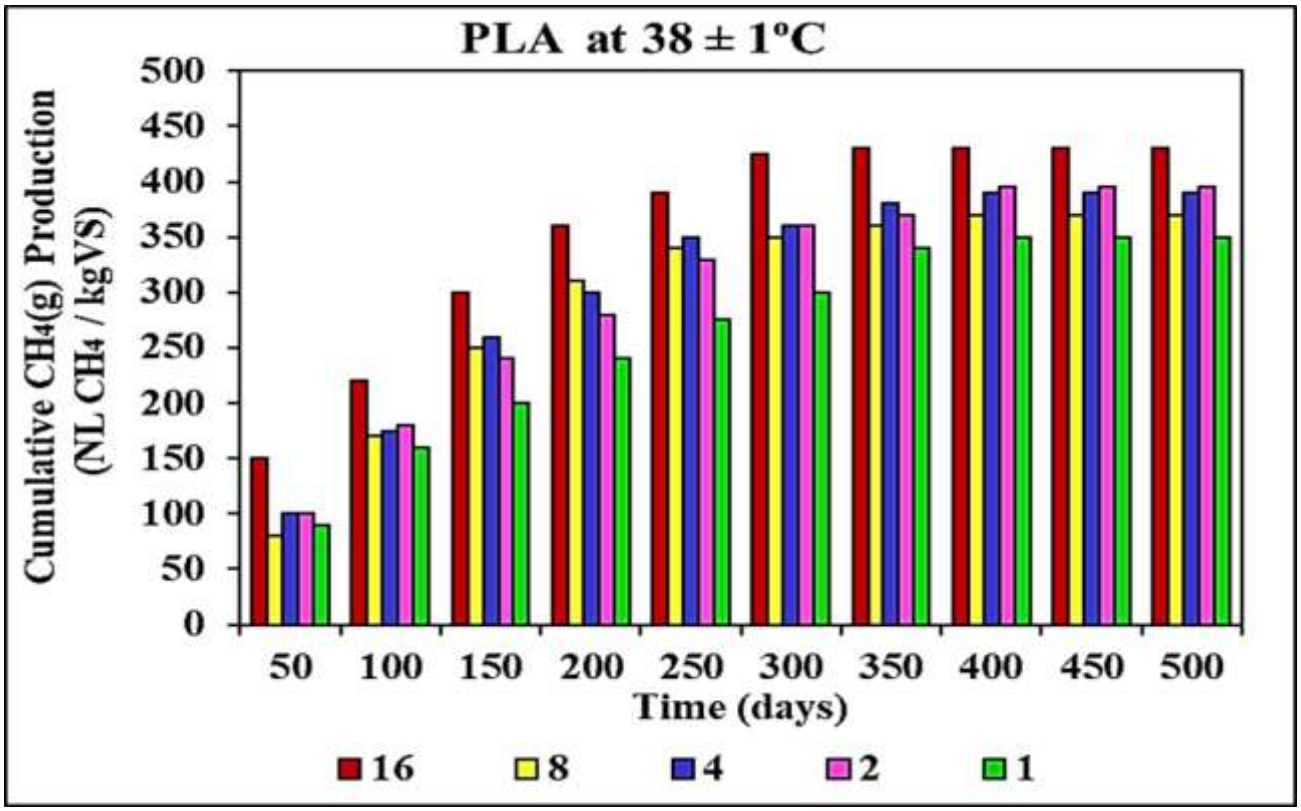


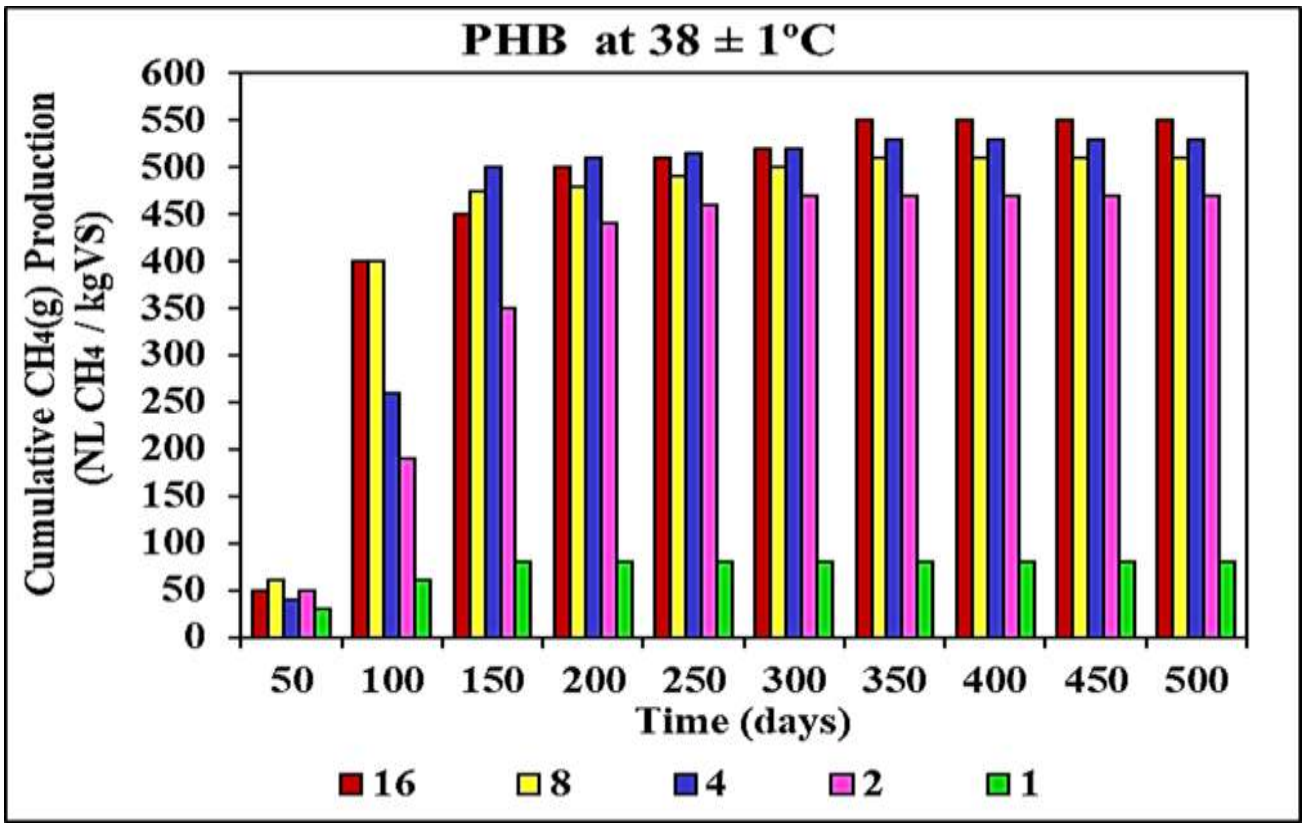
Fig. 1. The flow scheme of Anaerobic digestion process.

Table 3. The operational parameters of BMP tests (mean values ± standard deviation), (Cellulose was used as a positive control or blank samples).

Parameters	**T (ºC)**	**ISR (as VS based)**	**Mass of inoculum (g)**	**Mass of substrate (g)**	**Mass of water (g)**
Blank	38 ± 1	-	250.28 ± 0.16	0	50.87 ± 0.80
	58 ± 1	-	250.05 ± 0.05	0	50.13 ± 0.31
Cellulose	38 ± 1	-	250.24 ± 0.08	2.14 ± 0.02	48.09 ± 0.24
	58 ± 1	-	250.73 ± 0.29	1.37 ± 0.01	48.54 ± 0.21
PLA	38 ± 1	16	250.27 ± 0.19	0.63 ± 0	49.85 ± 0.43
		8	250.42 ± 0.29	1.56 ± 0.05	48.76 ± 0.18
		4	250.35 ± 0.46	2.16 ± 0.02	48.07 ± 0.09
		2	250.47 ± 0.18	3.04 ± 0.01	46.98 ± 0.11
		1	250.38 ± 0.14	6.14 ± 0.05	44.42 ± 0.49
PHB	38 ± 1	16	250.31 ± 0.17	0.61 ± 0.01	49.70 ± 0.25
		8	250.50 ± 0.19	1.52 ± 0.01	48.63 ± 0.05
		4	250.42 ± 0.36	2.10 ± 0.01	48.17 ± 0.29
		2	250.08 ± 0.05	2.99 ± 0.01	47.49 ± 0.27
		1	250.72 ± 0.10	6.10 ± 0.01	44.28 ± 0.09
PLA	58 ± 1	16	250.36 ± 0.27	0.51 ± 0.01	49.52 ± 0.05
		8	250.86 ± 0.80	1.75 ± 0.01	48.60 ± 0.11
		4	250.28 ± 0.01	1.79 ± 0.01	48.05 ± 0.04
		2	250.07 ± 0.21	2.57 ± 0.01	47.57 ± 0.01
		1	250.21 ± 0.58	5.03 ± 0.01	45.94 ± 1.43
PHB	58 ± 1	16	250.34 ± 0.17	0.58 ± 0.01	49.68 ± 0.22
		8	250.53 ± 0.18	1.54 ± 0.01	48.60 ± 0.04
		4	250.40 ± 0.31	2.07 ± 0.01	48.09 ± 0.27
		2	250.01 ± 0.05	2.81 ± 0.01	47.57 ± 0.27
		1	250.42 ± 0.11	5.98 ± 0.03	44.39 ± 0.09

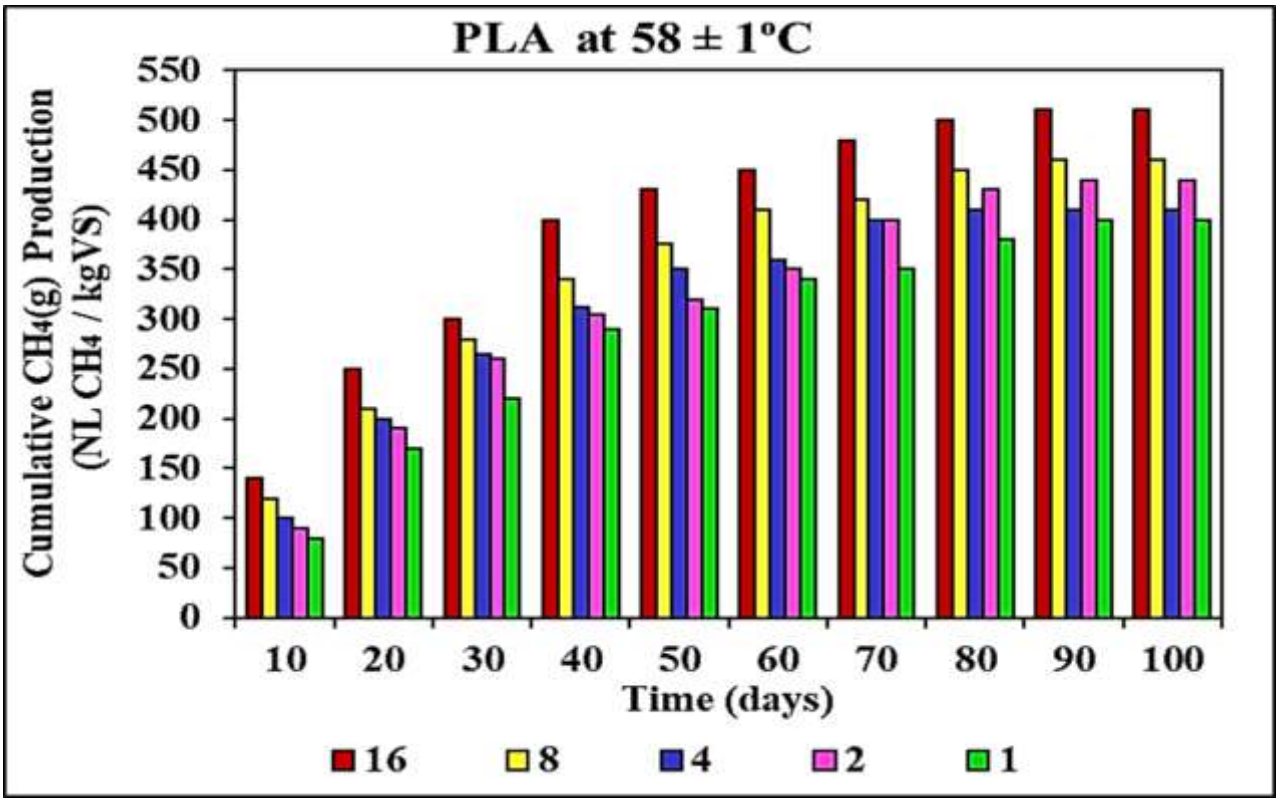


(a)

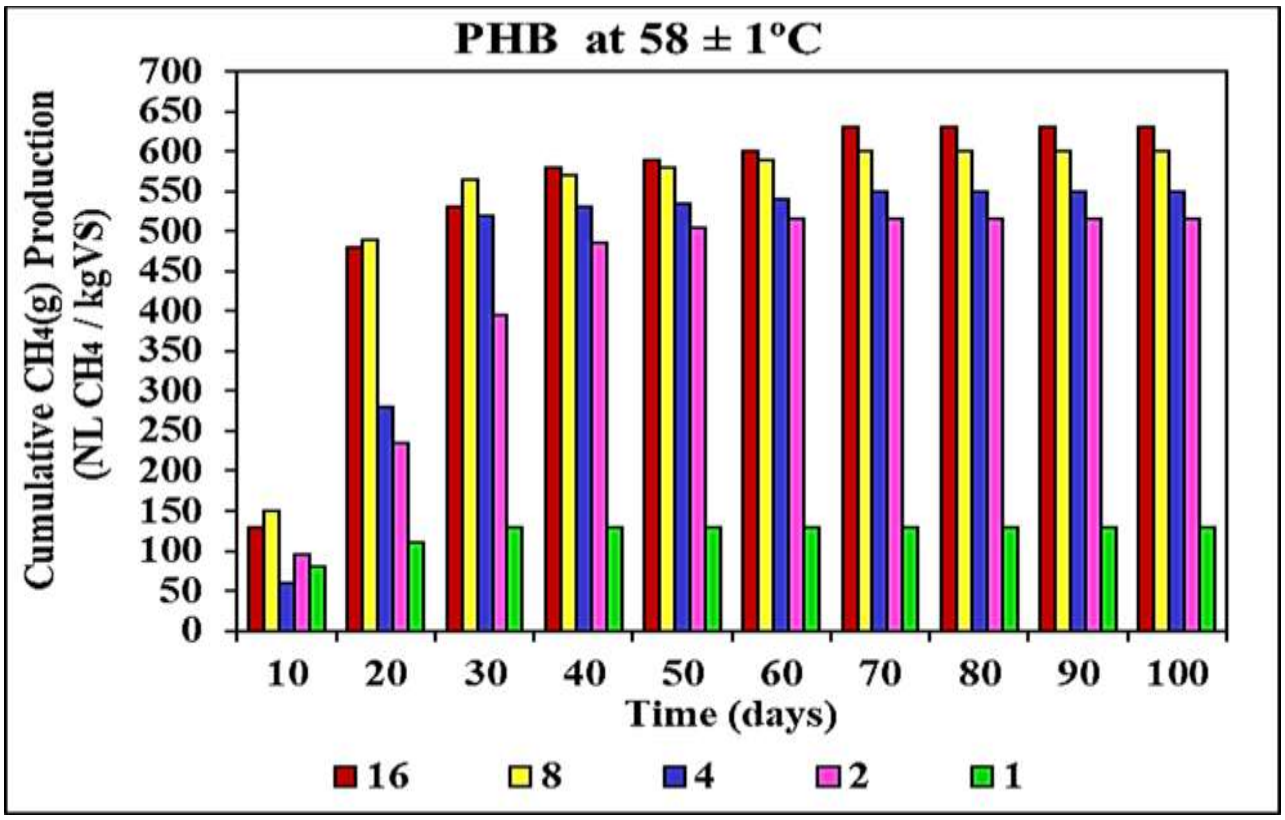


(b)

Fig. 2. The mean cumulative methane production from the various ISRs (16, 8, 4, 2 and 1) tested for (a) PLA under mesophilic conditions at pH=7.6. and (b) PHB under mesophilic conditions at pH=8.1, after 500 days and at 38±1°C.

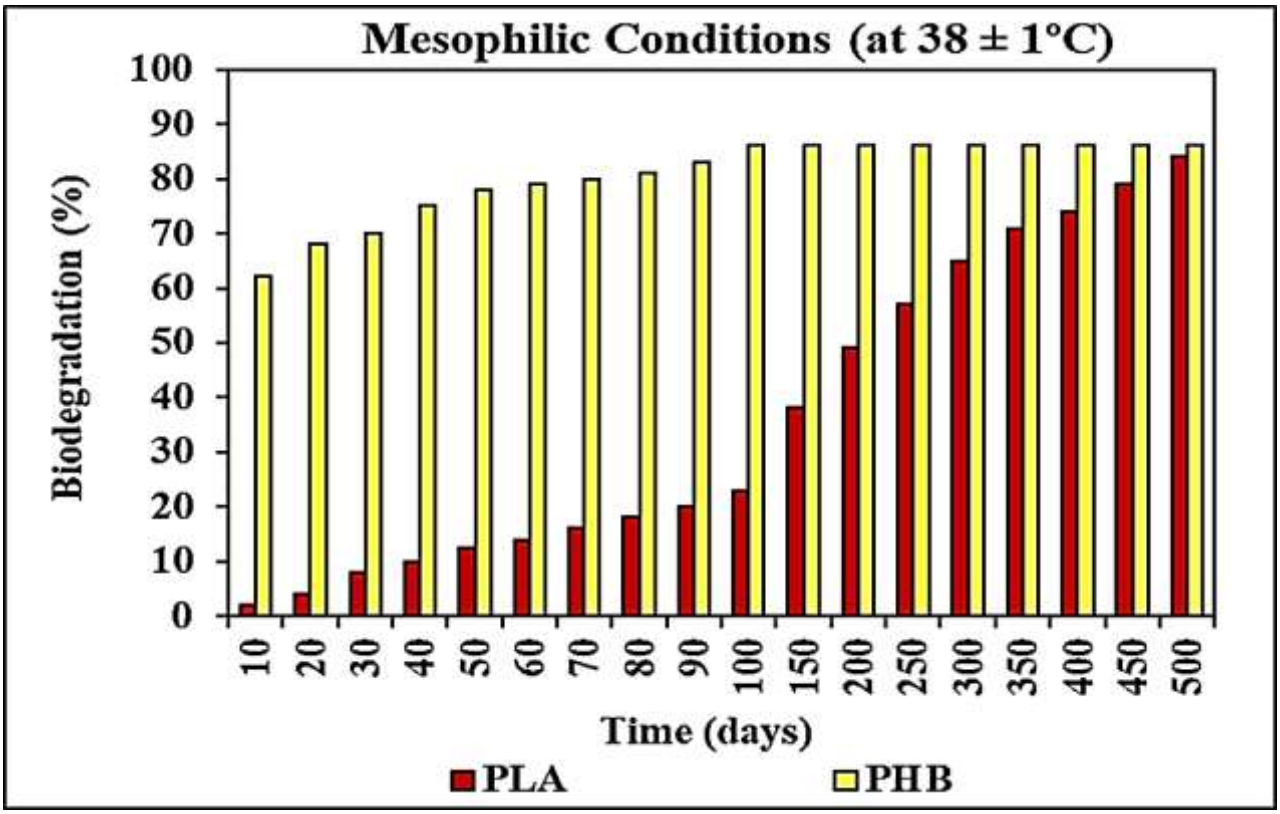


(a)

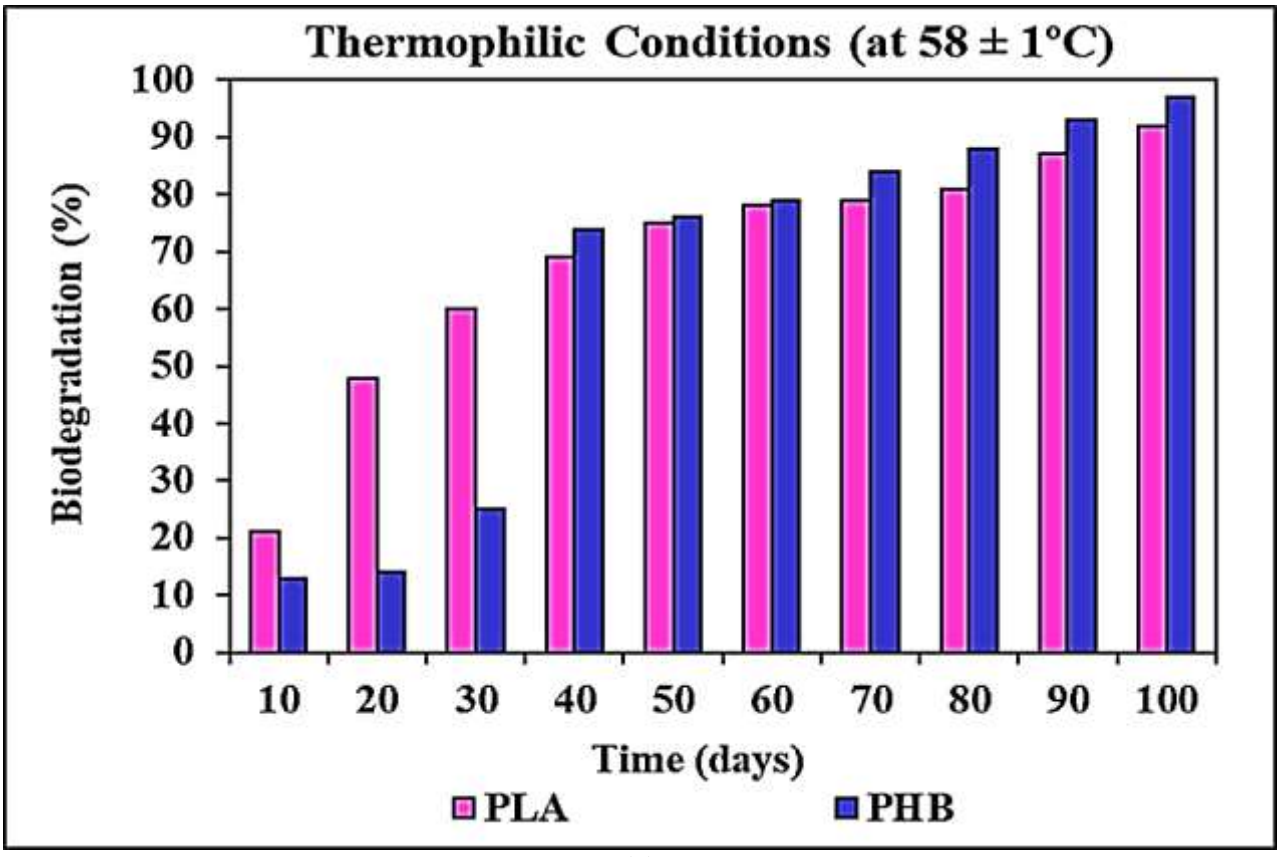


(b)

Fig. 3. The mean cumulative methane production from the various ISRs (16, 8, 4, 2 and 1) tested for (a) PLA under thermophilic conditions at pH=7.6 and (b) PHB under thermophilic conditions at pH=8.1, after 500 days and at 38±1ºC.



(a)



(b)

Fig. 4. The average biodegradation efficiencies based on theoretical methane production were obtained from PLA biodegradable plastics at pH=7.6 and PHB biodegradable plastics at pH=8.1 under (a) mesophilic (at 38 ± 1°C) conditions after 500 days and (b) thermophilic (at 58 ± 1°C) conditions after 100 days, respectively.

Table 4. The Monod-Gompertz modelling parameters of experimental data (mean values ± standard deviation), (Cellulose was used as a positive control or blank samples).

Parameters	**T(ºC)**	**ISR**	**λ Lag Phase (days)**	**G0 CH_4(g) Potential (NL CH_4/kg VS)**	**R_{max} CH_4(g) Production Rate (NL CH_4/kg VS.d)**	**R^2**
Cellulose	38 ± 1	2.85	1.89 ± 0.17	324 ± 15.10	63.27 ± 0.71	0.999
	58 ± 1	2.85	0.96 ± 0.08	335 ± 16.52	137.19 ± 7.94	0.999
PLA	38 ± 1	16	0	438 ± 15.22	1.97 ± 0.22	0.998
		8	0	397 ± 2.21	1.52 ± 0.04	0.997
		4	0	414 ± 4.27	1.56 ± 0.01	0.995
		2	0	429 ± 12.62	1.45 ± 0.03	0.997
		1	0	417 ± 4.56	1.29 ± 0.02	0.999
PLA	58 ± 1	16	0	468 ± 60.19	11.41 ± 0.84	0.996
		8	0	435 ± 10.24	9.19 ± 0.26	0.993
		4	0	401 ± 12.08	8.38 ± 0.52	0.989
		2	0	415 ± 19.76	7.52 ± 0.16	0.991
		1	0	386 ± 19.21	6.69 ± 0.01	0.994
PHB	38 ± 1	16	6.74 ± 0.13	529 ± 17.03	152.57 ± 15.17	0.997
		8	6.49 ± 0.05	503 ± 5.29	133.61 ± 0.29	0.998
		4	6.67 ± 0.15	538 ± 36.11	91.25 ± 6.78	0.999
		2	6.21 ± 0.51	511 ± 4.28	52.96 ± 27.41	0.994
		1	12.71 ± 1.27	61 ± 33.74	12.91 ± 15.60	0.992
PHB	58 ± 1	16	5.51 ± 0.13	584 ± 17.03	978.24 ± 98.16	0.998
		8	5.26 ± 0.05	545 ± 5.29	873.37 ± 80.32	0.998
		4	5.44 ± 0.15	522 ± 36.11	730.01 ± 69.11	0.999
		2	5.03 ± 0.51	536 ± 4.28	370.72 ± 39.27	0.996
		1	10.50 ± 1.02	42 ± 22.40	77.46 ± 25.09	0.993

Table 5. The comparative summary of error analysis results with our study and artificial neural networks (ANN) example approach (MAE: mean absolute error, RMSE: root mean square error, BE: bias error, Gap-fill ranges represent the ensemble of budgets derived from bootstrapped datasets using the respective gap-filling method).

Gap-fill method	**CH_4(g) analyser**	**MAE (nmol/m^2.s)**	**RMSE (nmol/m^2.s)**	**BE (nmol/m^2.s)**	**R^2**	**Cumulative flux (g-CH_4/m^2)**	**Gap-fill range**	**Relative gap-fill (%)**
In this study	TGA	9.6	14.2	0.25	0.96	63.1	62.7 – 63.6	1
ANN	TGA	7.8	10.4	0.19	0.99	62.9	62.5 – 63.4	5
In this study	LI-7700	8.8	11.3	0.12	0.98	64.7	64.3 – 72.2	2
ANN	LI-7700	8.5	9.5	0.11	0.99	64.3	64.0 – 64.8	2