Chapter 6

Recovery of Biodegradable Bioplastics from Different Activated Sludge Processes during Wastewater Treatment

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Abstract

To overcome the environmental hazards of petroleum based plastics, synthesis and use of microbial bioplastics became popular. Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers having plastic like properties mainly used in tissue engineering and packaging. Bacteria can produce bioplastics in carbon abundance. Activated sludge process is a simultaneous process for treating wastewater and producing PHAs. Wastewaters are treated by using mixed sludge, aerobic granular sludge and chemically treated sludge which provided more than 40% PHA yield. This chapter describes the PHA structure, synthesis pathways, types of wastewaters and activated sludge processes used with reactor parameters and environmental factors effecting PHA productions.

Keywords

Polyhydroxyalkanoates, Activated Sludge, Wastewater, Pathways, Synthesis Conditions, Extraction, Quantification

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1. Introduction

The ever-expanding production and extensive use of petroleum based plastics are one of the major environmental concerns of modern civilization. Starting from packaging to utensil, transport, furniture, pipelines and in different industrial sectors plastic usage has

become an integral part of our daily life. Polyamides, polyethylene, polyester, polypropylene, polyvinyl chloride (PVC) are the most commonly used plastic polymers nowadays. However, non-biodegradability, slow environmental accumulation and carbon dioxide (CO₂) emission are the major threats of plastic pollution [1]. Generation of approximately 34 million tons of plastic wastes has been reported throughout the globe and about 93% of them are disposed in oceans and utilized in landfill purposes [2].

Synthesis of bio-based biodegradable bioplastics is the environmentally viable alternative to fossil fuel based plastics. First bioplastic was produced and commercialized by Imperial Chemical Industries in 1980s under the trade name 'Biopol'. Polyhydroxyalkanoates (PHA) are polyesters with thermoplastic properties largely known as biodegradable biopolymer. Among all the PHAs, polyhydroxybutyrate (PHB) and its copolymer polyhydroxyvalerate (PHV) combine together to form a biocompatible polymer (poly(3-hydroxybutyrate-co-3-hydroxyvalerate)) known as PHBV[3]. Microbes are capable of producing PHA in abundance of external carbon source (feast phase) and store them for future use in absence of food (famine phase) and further convert it into energy for metabolic activities [4]. According to the literature, *Ralstonia eutropha* mediated and volatile fatty acid (VFA) enhanced pathways are the main proposed pathways for bioplastic production. Halophilic bacteria *Haloferax mediterranei*, *Ralstonia eutropha*, and genetically engineered recombinant *Bacillus subtilis* str. pBE2C1 and *Bacillus subtilis* str. pBE2C1AB are largely reported PHA producing strains [5]. Genetically modified bacteria were reported to accumulate more than 90% PHA of their cell dry weight by utilizing sugar substrate [6]. But to avoid the use of expensive pure culture bacteria and sugar substrates, activated sludge and wastewater can be used as a simple, renewable and low cost method of bioplastics production. Pie charts of Figure 1 [5] are describing the comparisons of global bioplastic market and traditional plastic markets.

The latest research trend in wastewater bio-refineries (WWBR) is driving the researchers' attention towards resource recovery during wastewater treatment. Wastewaters are considered of having more than 50% of the lost resources [6]. Hence, complex wastewater can be used as C, N, P (nutrient) enriched renewable feedstock for bacterial growth and precursor of PHA synthesis. Activated sludge process is a well known biological wastewater treatment used in both municipal and industrial wastewater treatment. This process involves the use of large amount of bio-sludge which can simultaneously treat wastewater and can recover renewable biomaterials.

Activated sludge based PHA production has been extensively studied mainly in palm oil mill effluent (POME), paper and pulp mill wastewater, dairy wastewaters, molasses spent wash and olive oil mill wastewater containing recalcitrant pollutants [4, 7-10]. Variation

in reactor parameters and conditions has significant impact in PHA yield. For microbial mixed cultures, aero dynamic feeding (ADF) strategy and regulation in feast famine regime and application of selection pressure enhanced PHA yield. However, sludge modification was proved to be fruitful in enhancing the PHA production. Several scientists conducted PHA extraction from different activated sludge like secondary municipal wastewater sludge, dissolved oxygen (DO) controlled municipal sludge, chemically treated and aerobic granular sludge [4, 11, 12].

Hence activated sludge process is an eco-friendly and economic process for environmental remediation as well as biodegradable bioplastic production leading to circular economy.

GLOBAL PLASTICS MARKET

- Bioplastic market expected to grow at **30% CAGR 2013-2030**
- Traditional plastics expected to grow 3% annually

Figure 1 The pie charts are depicting the global scenario for oil-based traditional plastics and bio-based bioplastics which is indicating that bioplastics hold 40% market share [5].

2. Chemistry and application of bioplastics

Polyhydroxyalkanoates are environment friendly, non-toxic biocompatible polyesters which are almost biodegradable in nature. Bacterial PHA can be produced by bacteria or haloarchaea in absence of carbon and nutrient sources. They are thermoplastics having very high melting point and degradation temperature of 179 °C [3]. PHAs are ultraviolet (UV) stable bioplastics soluble in chloroform. Their strength and flexibility can be modified by varying valerate percentages [5].

The structure and length of the PHA polymer chain depends on the type of microorganism and carbon source used for the synthesis, where polyesters are connected by ester bonds. PHAs generally carry one pendant alkyl group (R) in it where the R can vary from methyl (C₁) to tridecyl (C₁₃) [13]. However, PHAs contain a minimum of 100 and maximum of 35000 hydroxyacid (HA) monomers in it. The HA monomers generally prevail in three forms: 3, 4 and 5 hydroxyalkanoates [14]. They can be further divided into three classes on the basis of the length of their side chain. They are

- Short chain length PHAs (scl-PHAs) containing short chains having 3 to 5 carbons. They have rigid crystalline structure,
- Medium chain length PHAs (mcl-PHAs) possess medium length chains containing minimum 6 and maximum 14 carbons having a soft and sticky characteristics and
- Large-chain-length PHAs (lcl-PHAs) are having 15 or more carbons [15].

PHAs are mostly used in packaging and coatings. Due to the unique biocompatibility properties, PHAs are also largely being used in pharmaceutical and medical usage like wound bandage, biomedical devices like bone scaffolds (used in tissue engineering implants like heart valves), orthopedic pins, heart stents, nerve guides etc. Moreover, there are also several other applications of bioplastics like in making electronic gadgets (cell phone), encapsulation, crop protection by plastic films, diaper making (personal hygiene) and in formulating adhesives [16].

3. PHA synthesis pathway

The most well-known PHA production pathway is given in Figure 2 [5]. *Ralstonia eutropha* mediated PHA synthesis pathway is used in industrial scale production [17]. Generally, scl-PHAs are produced in this process. The biosynthesis is initiated with the conversion of sugar substrate or organic acids to acetyl coenzyme-A (acetyl-CoA) via glycolysis and beta-oxidation. β -ketotiolase (PhaA) enzyme helps in the condensation of two molecules of acetyl-CoA to produce acetoacetyl coenzyme-A. It is further converted to (R)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB) by reducing nicotinamide adenine dinucleotide phosphate (NADPH) to NADP⁺. Finally, 3-

hydroxybutyryl-CoA monomer is polymerized into P(3HB) by PHA synthase enzyme (PhaC).

Figure 2 The first discovered pathway for PHA biosynthesis [5].

Mixed microbial culture (MMC) mediated PHA biosynthesis is generally described in 3 following steps [18].

- In *Step 1* organic sugar substrate is converted into VFA enriched fermented product.
- In *Step 2* sequencing batch reactor (SBR) is operated with optimized operating conditions and microenvironment to select the PHA accumulating microorganisms among the mixed culture.

- *Step 3* is the final stage where the biomass is cultivated with VFA feeding which resulted into PHA production under optimal conditions. The biosynthesized PHA is further harvested by downstream processing of extraction.

4. Activated sludge process for PHA production

The microbial diversity of activated sludge made that process suitable for bioplastic synthesis. Modified sludge processes are proved to be more efficient in bioplastic yields. Mixed, aerobic granular and chemically treated sludge processes are described below.

4.1 Mixed activated sludge process

Wen and co-workers [19] used bulking sludge for PHA production. They operated two SBRs , one with aerobic dynamic feeding (ADF) model (R2) and another one by anaerobic phase followed by an aerobic phase (R1) in order to find out mixed cultures with high PHA accumulating capacities. Due to the incorporation of anaerobic phase before aerobic phase, in R1 sludge settleability was improved but the simultaneous increase in feast/famine ratio was responsible for less PHB accumulation in R1 than R2. Maximum PHB yield was 53% of the total suspended solids which was achieved at 10.2 h of a batch study under the starvation of nitrogen. The study indicated that filamentous bacterial sludge can also accumulate PHB while compared to the well settled sludge. The low oxygen requirement of the filamentous sludge helped to save energy required for PHA producing bacterial enrichment.

PHA production was obtained in a three-step process utilizing food waste as a renewable source [20]. In step I, bio-hydrogen was produced in acidogenic fermentation pathway. The VFA enriched effluent produced from bio-hydrogen reactor was utilized for PHA production. PHA production occurred in two stages. Step II was conducted for microbial enrichment and finally step III was for PHA production. PHA producing bacteria were cultivated inside a SBR , which was operated in two different cycles of CL-24 and CL-12. Maximum PHA recovery was achieved in CL-12 cycle in both Step II (PHA yield: 16.3% of dry cell weight with 84% VFA removal) and Step III (PHA yield: 23.7%; 88% VFA removal). The synthesized PHA obtained was basically in a copolymer form of [P(3HB-co-3HV)] of PHB and PHV. Hence this study established an innovative approach of using wastewater in simultaneous waste remediation and bioplastic production by using mixed culture sludge.

In another literature PHA extraction was reported by using municipal secondary wastewater sludge by improving different parameters like temperature, operation time and solid biomass concentration [21]. After parameter optimization, maximum PHA

production was 0.605 g which was higher than the un-optimized operating conditions. The produced PHA was mostly dominated by 58% mcl PHA which was produced in comparatively low cost than the conventional bioplastics. Again, this study focused on the probable low cost biomaterial production by using waste resource with simultaneous waste minimization.

Different PHA copolymers were extracted from municipal activated sludge cultivated in fermented waste feedstock [11]. In biological wastewater treatment, nitrification occurs first where ammonia is converted to nitrate by ammonia and nitrite oxidizing bacteria (AOBs and NOBs) and finally it is removed as nitrogen via denitrification. Activated sludge can accumulate PHA along with nitrogen removal. Dissolved oxygen (DO) had a significant role in regulating PHA production and nitrification of the activated sludge process. The experiment finally suggested a PHA recovery method by utilizing nitrifying activated sludge where DO level should be maintained while (1) NOB growth and activity are mitigated, (2) nitrogen removal is observed, (3) alkalinity is controlled by denitrification, and (4) aeration is reduced.

4.2 Aerobic granular sludge process

Aerobic granulation technology is one of the most popular biological wastewater treatment strategies in current research trends for sustainable environment. Presence of microbial diversity, formation of even shaped compact sludge granules with high settling velocity and recalcitrant pollutant removal efficiency made this process significant for the researchers. Different types of domestic as well as industrial wastewaters have been successfully treated by aerobic granular sludge so far. However, many literatures have also reported the PHA accumulation by aerobic granules while treating the wastewater in aerobic granular reactors (AGR). Table 1 [4,7, 22-24] provides the literature details of aerobic granulation mediated PHA production while treating different wastewater with operating conditions, granule characteristics and PHA yield.

In Figure 3 [7] the PHA accumulation inside aerobic sludge granules is visible in different famine-period aeration rate of 2 L/min, 1 L/min, 0.5 L/min and 0 L/min, respectively.

Table1 Aerobic granulation in different wastewater treatment with different operating conditions, granule characteristics and PHA yield [4,7, 22-24]

Wastewater	Operating condition	Granule characteristics	Pollutant removal efficiency	PHA yield	Ref.
Acetate wastewater (synthetic)	AGR volume: 2 L, HRT: 12 h, cycle time: 6 h, settling time: 2 min, Influent COD: 500-1000 mg/L, ammonia nitrogen: 25-12.5 mg/L, COD/N: 20-90	MLSS: 4-8 g/L, MLVSS: 4.5-10 g/L	COD: 80-99%	44%	[22]
Acetate wastewater (synthetic)	AGR volume: 2 L, HRT: 12 h, SRT: 10 days	MLVSS: 3-9 g/L, SVI: <40 mL/g, EPS: 6-30 mg/g VSS	COD: 90%	40 ± 4.6%.	[23]
Palm oil mill effluent (POME)	Cycle time: 6 h, settling time: 1.5 min, VER: 25%, H/D: 10, air flow rate: 3 L/min, COD: 51,000 mg/L	EPS: 40–45 µg/mL	COD: 90%, propionic and butyric acids: 100%	0.6833 mg PHA/mg biomass.	[4]
Palm oil mill effluent (POME)	AGR volume: 2L, VER: 25%, cycle time: 24 h, air flow rate: 3 L/min, OLR: 0.91, 1.82, 2.73 and 3.64 kg COD/m ³ day.	Granule size: 0.35-2 mm	COD: 90%	0.66 to 0.87 g PHA/g CDW	[24]
Palm oil mill effluent (POME)	AGR volume: 2 L, cycle time: 6 h, air flow rate: 3 L/min	Granule size: 1.03 mm, SVI: 20-40 mL/g	COD: 90%	0.56 g PHA/g CDW	[7]

HRT: Hydraulic retention time, **MLSS:** Mixed liquor suspended solids, **MLVSS:** Mixed liquor volatile suspended solids, **VSS:** Volatile suspended solids, **COD:** Chemical oxygen demand, **EPS:** Extracellular polymerix substances, **H/D:** Height/diameter ratio, **SRT:** Sludge retention time, **VER:** Volume exchange ratio, **SVI:** Sludge volume index, **CDW:** Cell dry weight.

Figure 3 PHA accumulation inside aerobic sludge granules is visible in famine-period aeration rate of (a) 2 L/min, (b) 1 L/min, (c) 0.5 L/min and (d) 0 L/min [7].

4.3 Chemically modified sludge process

Currently, chemically enhanced primary sedimentation (CEPS) technique is extensively being used in Hong Kong as an effective sewage treatment process. The CEPS sludge consists of various organic compounds which can recover VFAs which can be further utilized for PHA production. Xu and co-workers [12] observed that addition of CaO_2 enhanced the disintegration of CEPS sludge and rapid VFA production occurred. The maximum VFAs yield was 455.8 mg COD/g VSS and the microbes requiring hydrolysis and acidogenesis were enriched with the VFA. CaO_2 treatment had no influence on the release of ammonia nitrogen ($\text{NH}_4^+\text{-N}$), but it proved to be significant in PHA biosynthesis by sludge microbes. The VFA enriched feedstock proved to be a suitable substrate for PHA production. After the cultivation phase in VFA enriched substrate the CEPS sludge achieved almost 22.3% PHA accumulation.

5. Types of wastewaters in bioplastic production

5.1 Palm oil mill effluent (POME)

Palm oil mill wastewaters are enriched with VFAs. Aerobic granulation mediated PHAs were generally produced by using POME where more than 90% influent COD was removed by complete removal of VFAs [4]. Maximum 0.66 to 0.87 g PHA/g CDW PHA accumulation was reported by Vjayan and Vadivelu [7].

5.2 Paper pulp mill wastewater

PHA production was also carried out by using paper mill wastewater [8]. The biosynthesis process was carried out into the following steps. (1) A single batch process was required for the acidogenic fermentation of the paper mill wastewater, (2) PHA producing bacterial enrichment in batch mode operation in a feast-famine regime, (3) maximizing the PHA yield by PHA accumulating strain enrichment in batch operation, and finally (4) the selective pressure was also required for microbial enrichment. However, the study resulted into 77% yield of PHA of cell dry weight within 5 h of batch study after microbial enrichment.

5.3 Dairy wastewater

Dairy wastewater activated sludge was used as the inoculums to produce PHA by using milk whey [9]. The biosynthesis was carried out into several steps by pre-treating the sludge to reduce protein concentration, optimizing a suitable carbon/nitrogen (C/N) ratio for maximum PHA yield and then pH correction for synthesis. The study showed that at a

C/N=50 about 13.82% PHA yield was possible without pH correction. Another advantage of using dairy wastewater was no requirement of aseptic condition.

5.4 Molasses spentwash

A potential low cost feedstock molasses spent wash was utilized by waste activated sludge bacteria to produce biodegradable biopolymer polyhydroxybutyrate (PHB) [25]. The synthesized PHB was visible inside sludge biomass under fluorescence microscopy. PHB was further characterized by fourier transform-infra-red spectroscopy (FT-IR) and ^{13}C nuclear magnetic resonance (NMR). At C:N=28 condition, maximum 52% COD removal was achieved with maximum PHB accumulation of 28%.

5.5 Olive oil mill wastewater

Olive oil mill wastewater was used as a no-cost substrate for PHA accumulation in a mixed microbial culture [10]. The process was carried out in multistages with phenol removal and recovery. Initially phenol was removed from the wastewater and selection of the PHA-producing bacteria was carried out inside an SBR. At optimum influent organic loading rate (OLR) of 4.70 g COD/L.d maximum PHA storage and yield was observed. Mass balance indicated that about 85% of the influent COD was converted into 10% PHA (volumetric productivity). Maximum PHA yield was found as 1.50 g PHA/L.d.

6. Conditions for PHA production

6.1 Aerodynamic feeding (ADF)

Aerodynamic feeding (ADF) is a very popular feeding strategy for PHA production from mixed sludge. ADF helps in establishing a strong feast-famine regime in a SBR cycle which help the microorganisms to accumulate PHA in abundance of carbon source (feast phase).

Amulya and coworkers [20] employed ADF in a three-step process for PHA production from microbial mixed culture (MMC). In first step, bio-hydrogen was produced in acidogenic fermentation. In second step, VFA enriched substrate enriched the PHA producing microorganisms and in 3rd step finally PHA synthesis was obtained. The final product was a copolymer of [P(3HB-co-3HV)] of PHB and PHV.

Gobi and Vadivelu [4] were able to produce maximum 0.6833 mg PHA/mg biomass from aerobic granular sludge while treating palm oil mill effluent (POME).

6.2 Feast-famine regime

According to literature, during PHA production from activated sludge, high oxygen consumption was required to oxidize the external carbon source in feast phase. But in famine phase less oxygen is required for the oxidation of stored PHA and to convert it into metabolic energies. However, the reduction in oxygen consumption contributes to energy and cost reduction in commercial PHA production. Hence feast-famine regime of a reactor cycle plays an important role in PHA accumulation by mixed microbial culture.

By regulating feast-famine regime and uncoupled carbon and nitrogen, Oliveira and coworkers [26] were able to produce 6.09 gPHA/L of PHA from mixed microbial culture by utilizing cheap cost industrial and agricultural waste feedstock.

6.3 pH

Impacts of microenvironment and pH values were also evaluated in PHA production from wastewater [27]. The study was conducted by varying the SBR microenvironment from aerobic to microaerophilic and also by regulating the reactor pH as 6, 7 and 8. The study showed that neutral pH with microaerophilic reactor environment was more favourable for PHA production than other conditions.

6.4 Carbon/nitrogen ratio

High COD loading and low ammonia concentration was proved to be very effective in high PHA yield from activated sludge. Hence, selection of an optimum C/N ratio was important to enhance PHA production.

Fang et al. [22] observed that at 750 mg/L of COD and 8.5 mg/L of influent ammonia concentration maximum 44% PHB production was obtained from aerobic granules while treating synthetic acetate wastewater. The study also revealed that increasing ammonia concentration enhanced granule biomass concentration and settling velocity but reduced the PHB accumulation. So, it was suggested that by regulating influent ammonia concentration we can obtain maximum PHB yield with cultivating highly settleable compact sludge granules.

In another study, dairy wastewater activated sludge was used as the inoculum to produce PHA by using milk whey [9]. Finally, the study showed that at C/N=50 about 13.82% PHA yield was possible without pH correction.

6.5 Selective pressure

Chen et al. [28] proposed a novel approach of aerobic dynamic discharge (ADD) to provide physical selective pressure to enhance PHA production in PHA accumulating

bacteria in a system having MMCs. The study provided a comparative analysis of ADD coupled with SBR operation with conventional aero dynamic feeding (ADF) method. Microbes treated with ADD mode resulted into maximum PHA yield of 0.72 ± 0.07 Carbon mol PHA/Carbon mol acetate and maximum PHA content of $74.16 \pm 0.03\%$ of biomass weight after 30 days of the ADD operation, which was higher than MMC operated with ADF mode. The study provided a new ADD strategy for high PHA accumulation possibility in SBR mediated MMC system.

7. PHA extraction process

PHA extraction methods can be classified into 2 categories.

- Chemical digestion with solvent extraction and
- Mechanical disruption

7.1 Chemical digestion with solvent extraction

Sodium hypochlorite, acetone, chloroform and sodium hydroxide are commonly used chemicals for PHA extraction. Figure 4 [29] provides a flowchart for aerobic granular biomass digestion and PHA recovery from granules by using four chemicals acetone, NaOCl, NaCl and NaOH.

Figure 4 This flowchart shows aerobic granular biomass digestion and PHA recovery from granules by using four chemicals acetone, NaOCl, NaCl and NaOH [29].

7.1.1 Sodium hypochlorite extraction

About 12.5 mL sodium hypochlorite and 12.5 mL of chloroform is required for PHA extraction from 1 g of lyophilized biomass. After vortexing the mixture is incubated at 37° C temperature for 90 min and then after centrifugation phase separation is observed. The top layer having sodium hypochlorite is discarded and the chloroform layer containing PHA is collected for recovery [30].

7.1.2 Acetone extraction

For 1 g of lyophilized biomass, about 12.5 mL acetone is required to remove cell impurities. The mixture is kept at 65° C temperature and then the acetone layer is discarded and the biomass is subjected to PHA dissolving step [31].

7.1.3 Sodium hydroxide extraction

About 1 g of lyophilized biomass is digested by using 0.05 M NaOH. Finally, the biomass is immersed in 6.84 N concentrated sodium chloride solution inside a shaker incubator for 24 h. The sodium chloride layer is discarded and the biomass is subjected to PHA recovery [32].

7.2 Mechanical disruption

Mechanical disruption is generally carried out by providing high pressure bead milling or homogenization without using any chemicals. However, there are several drawbacks of this method [33]. They are:

- Cell damage due to mechanical pressure
- Long process time is required
- More power consumption
- Difficult to scale up the process.

8. Quantification method

After the extraction of PHA it is dried up and the resultant weight is recorded. The PHA yield is calculated by comparing with total cell dry weight [4]. The PHA yield is expressed by Eq. 1

$$Y_{PHA/CDW} = \frac{\text{Total amount of PHA,mg}}{\text{Cell dry weight,mg}} \quad (1)$$

In many literature PHA yield was also expressed with respect to COD consumption [4]. It is calculated according to Eq. 2.

$$Y_{PHA/COD} = \frac{\text{Total amount of PHA,mg}}{\text{Total amount of COD converted,mg/L}} \quad (2)$$

Conclusion and future scopes

In modern scenario, environmental viability and cost effectiveness of bioplastics are increasing its market potential. Like agro, plant and algal-based bioplastics, bacterial bioplastics are also being studied throughout the world. Activated sludge process provides a simultaneous flow of waste management and biomaterial recovery. Activated sludge process involves the use of cheap or no-cost wastewater and waste sludge bacteria which can remove a big part of the production cost. Extensive studies have been conducted for PHA synthesis in activated sludge process by utilizing POME, dairy wastewater and paper mill wastewater due to presence of VFA which can be used to enrich PHA accumulating bacteria. Many researchers were able to enhance PHA synthesis by regulating different feeding strategies like ADF, ADD, by controlling feast-famine regime, microenvironment and pH of the reactor and also by varying the C/N ratio and DO concentration.

However, the major drawback of this activated sludge mediated PHA production is the lack of purity of the product. Hence, more selective sludge bacteria and modified extraction techniques should be implemented to ensure purity of the product. In PHA extraction, use of large amount of organic solvents is also not very economic and environmentally viable. More studies on recycling and reuse of solvents and investigation of low cost extraction process are required. Further studies should explore the probable utilization of innovative cheap cost feedstocks to ensure more economic PHA production. Moreover, for successful PHA production, scale-up from lab scale to pilot and industrial scale is required to establish activated sludge based bioplastic production market.

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