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A review on recovery of proteins from industrial wastewaters with special emphasis on PHA production process: Sustainable circular bioeconomy process development

Bhoomika Yadav[1], Shraddha Chavan[1], Anusha Atmakuri[1], R.D. Tyagi[1*], Patrick Drogui[1]

[1]INRS Eau, Terre et Environnement, 490, rue de la Couronne, Québec G1K 9A9, Canada

**Corresponding author: Tel: 1-(418) 654-2617; Fax: 1-(418) 654-2600. Email:Rd.tyagi@ete.inrs.ca*

Abstract

The economy of the polyhydroxyalkanoate (PHA) production process could be supported by utilising the different by-products released simultaneously during its production. Among these, proteins are present in high concentrations in liquid stream which are released after the cell disruption along with PHA granules. These microbial proteins can be used as animal feed, adhesive material and in manufacturing of bioplastics. The recycling of the protein containing liquid stream also serves as a promising approach to maintain circular bioeconomy in the route. For this aim, it is important to obtain good yield and limit the drawbacks of protein recovery processes and associated costs. The review focuses on recycling of the liquid stream generated during acid/thermal-alkali treatment for PHA production that would close the gap in linear economy and attain circularity in the process. Examples to recover proteins from other industrial waste streams along with their applications have also been discussed.

Keywords: Polyhydroxyalkanoates; Circular bioeconomy; Protein recovery; Recycling; Waste streams.

1. Introduction

The modern industrial sector currently follows the linear economy principle which is built on the 'make, use and dispose' pattern (Stahel, 2016) that is unsustainable and there is a requirement of an alternative flow model, one which is cyclical. The circular economy approach is oriented in a way that it focusses on product, material reuse, repair, remanufacturing, and cascading as well as biomass and waste-derived energy utilisation during the product value chain (Korhonen et al., 2018).

Petroleum plastics are such materials that utilises the linear economy concept in their production and consumption. They are produced from petroleum, which being non-renewable resource, take thousands to millions of years to replenish. In addition, the excessive use of plastics is causing pollution in environment because of their non-biodegradable nature (Yadav et al., 2019). Ingestion of micro-plastics mistaken for food is well reported in fishes, turtles, sea birds, and marine mammals which can be fatal. This concern over fate of petrochemical plastics in environment has created an interest to develop alternate materials. Bioplastics such as polyhydroxyalkanoates (PHAs) are biodegradable plastics produced mainly by bacteria (Vu et al., 2020). PHAs are recognised with immense possible applications in decreasing waste problems associated with most plastics. They are attractive because of their biodegradable nature and properties similar to conventional plastics such as polypropylene (Sirohi et al., 2020; Vu et al., 2020). All these properties of PHA have made them next generation bioplastic however, PHA penetration in market is still very limited which is due to their high production cost. The ability to generate PHA from inexpensive and renewable sources of carbon can make the process cost-effective (Moita et al., 2014; Pernicova et al., 2019).

Improving the economy of the PHA production from wastes and maintaining a circular bioeconomy could give a boost towards its concrete industrial implementation. Recovering useful biomaterials such as polysaccharides, surfactants, pigments, carotenoids, amino acids and their derivatives, ectoines, hydrogen and bioelectricity in co-production with PHA has received a great attention recently (Kumar & Kim, 2018). It has been realized that the cell disruption process after the fermentation step of PHA releases organic matter of cells such as proteins, polysaccharides, nucleic acids, lipids and cell wall fragments. (Koller, 2015; Yadav et al., 2019). Generally, protein accounts around 50% of the dry weight of bacterial cells (Delamare-Debouttéville et al., 2019; Xiao et al., 2017) and thus, can be present in variable concentrations depending upon the biomass. More value can be added to PHA production process by recovering the components such as proteins released in the liquid stream after cell disruption or by recycling these liquid streams in the fermentation process itself as nutrient source for biomass growth and PHA production (Koller, 2015).

Both PHA and microbial proteins are intracellular in nature and are released simultaneously after cell disruption. After cell disruption methods such as alkaline-heat treatment, PHA is released in insoluble form which can be precipitated, purified and recovered. However, the microbial protein is present in soluble phase and can be efficiently and economically separated. Ideally, one should aim to obtain high-quality product with efficient recovery rate, combined with maximum removal of other contaminants using minimal plant investment at lowest costs (Mulder, 2010). Depending on the characterisation, the recovered protein could be used as animal feed, pet food, glue or as bioplastic component

that may also help to mitigate global energy shortage (Adhikari et al., 2018; Mekonnen et al., 2016; Pervaiz & Sain, 2011; Xiao et al., 2017).

Reuse of liquid streams of PHA production process containing microbial proteins can help in developing an environment-friendly PHA production process. The process will improve waste management and resource efficiency which is important to maintain a circular bioeconomy approach in the PHA process (Gajaria et al., 2017). Moreover, the microbial proteins recovered from the cells can help in decreasing the cost of PHA production, making process more economical (Adhikari et al., 2018). Therefore, the review attempts to analyse and summarize literature on protein precipitation and recovery from waste streams generated from various industries that could be applied to recover proteins from the liquid streams of PHA production process. Critical discussion on protein precipitation methods including their mechanisms, possible merits/demerits and applications of the recovered proteins have been provided. It is for the first time, the integration of circular bioeconomy into protein recovery from the waste streams of the PHA production process has been discussed based on the current research trends.

2. The need of circular bioeconomy

In 2015, 241 million tonnes of municipal solid waste was generated in European Union (EU) out of which around 40-60% was organic waste, containing high salt content and moisture which can lead to greenhouse gas emissions if not managed properly (Vea et al., 2018). However, this organic waste represents a great candidate for renewable energy production and value added products like bio-pesticides, organic fertilizers and bioplastics. The EU introduced the circular economy (CE) strategy to push out the dominant linear economic principles of "take, make and dispose" towards a circular resource management

attitude. In CE, the 'end of life concept' is replaced with restoration by minimising the material use, reusing and recycling materials in production or in consumption process, using renewable energy, eliminating the use of toxic chemicals, and shifting towards zero waste discharge through modifications in the designs of products, materials, systems and business models. Transition towards circular bioeconomy (CBE) is one of the most important methods for waste stream recycling to move towards a sustainable future. CBE deals with cascading processing of bio-residues into bio-based products and materials that can be shared, reused, remanufactured and recycled, or released to the biosphere by organic and nutrient cycles (Mohan et al., 2019).

CBE approach can be applied to PHA production by using wastes such as municipal and industrial wastes to provide a sustainable source of carbon and nutrients (required for microbial growth and PHA production) that will improve the resource efficiency, waste management and reduce the overall cost of PHA production (Pakalapati et al., 2018; Rathika et al., 2019; Reddy et al., 2019; Yadav et al., 2019). Incorporation of CBE in the PHA process would help in managing the liquid streams generated during production process, recovering by-products such as crude microbial proteins that are discarded as waste liquid streams, promoting the reuse and recycle concept in the production cycle and inculcating them in a full-chain course of production, distribution and consumption to maintain the economy of the PHA production process (Gajaria et al., 2017; Xiao & Zhou, 2019) (Figure 1). Unfortunately, the industrial technologies are currently focussed on improving the overall process but ignores together the 4'E' aspects of sustainable production i.e. 'Economic, Ethical, Environmental, and Engineering' impacts thus, overlooking the CBE approach that could be incorporated for a sustainable recovery of

value-added by-products other than PHA (Dietrich et al., 2017). Therefore, a closed cycle of the liquid stream recycling and/or recovery of other bio-products during the process need to be proposed.

#Insert Figure 1 here#

3. Current state of PHA and co-production of other bio-products

In recent years, many efforts have been done for large scale production of PHA whose applications cover a number of biological polyesters with properties ranging from thermoplastic to elastomers (Koller & Braunegg, 2018). Although the price of PHAs is high, several companies such as Biomer (Germany), PolyFerm (Canada), Danimer Scientific (USA), Kaneka Corporation (Japan), Tianjin GreenBio Materials (China), Tepha Inc. (USA), TianAn Biologic (China), and Bio-On (Italy) are producing PHA products worldwide to meet the demand of the market (Ryberg et al., 2019). Cost of bioplastics is greatly influenced by the cost of raw materials being used, in addition, it also has an effect on ecological pressure. Carbon sources used for conventional PHA production constitutes of pure carbohydrates like glucose, sucrose, fatty acids and its derivatives, alkanes and methanol. Around 50% of the total production costs is attributed from carbon sources (Aslan et al., 2016). Therefore, to improve its profitability and facilitate PHA implementation in the market industry, substitution of pure substrates with industrial by-products and/or the waste streams are considered and sincere efforts has been done to alleviate PHA synthesis cost (Kumar et al., 2016). It is reported in many studies that the utilization of substrates, such as lignocellulosic bio-wastes, crude glycerol from biodiesel industry, macroalgal biomass, waste cooking oil, sugar-rich biomass and cheese whey could reduce the PHA production cost (Ghosh et al., 2019; Kumar et al., 2018; Kumar et

al., 2016; Li et al., 2020; Morya et al., 2018; Pernicova et al., 2019; Sirohi et al., 2020). The production of PHA depends on various factors such as feedstock type, micro-organism used, pre-processing techniques, nutrients, and operating parameters (Li & Wilkins, 2020; Rodriguez-Perez et al., 2018). Some feedstocks (for example, lignocellulosic biomass) can be transformed into readily consumable carbon only after physicochemical or biological pre-treatments. Such pre-treatments increase the carbon source availability, dilute the organic matter concentration, regulate pH, sterilize waste materials, control temperature and minimize potential inhibitory effects on microbial strains (Li & Wilkins, 2020). Table 1 gives a brief idea about the various waste streams used as substrates for production of PHA.

#Insert Table 1 here#

4. Concomitant recovery of proteins with PHA production

4.1 Microbial protein containing streams with examples from industrial wastewaters

The liquid waste streams released has to be first characterised in terms of total nitrogen, crude protein content, amino acid composition, organic matter, metals, chemical oxygen demand (COD), and biochemical oxygen demand (BOD) in order to understand its nature and protein precipitation process. The waste streams from different industrial processes contains high BOD, COD and nitrogen content. For example, sericin wastewater from silk degumming process contained around 5000 mg/L BOD, 9000 mg/L COD, 0.11% nitrogen and 0.69% protein content. The protein recovery from such waste streams will reduce the organic load and thus, reduce the treatment costs associated with these liquid streams. In addition, the recovered protein may be sold as a commercial product that could support the process economy (Vaithanomsat & Kitpreechavanich, 2008). In leather tannery

wastewater, organic nitrogen mostly consists of proteins and amino acids. It is possible to generate proteins at costs taking in account the revenue from avoiding the treatment of mineral nutrients present in side streams (Matassa et al., 2016). The protein content in slaughterhouse waste streams ranged from 444 to 2775 mg/L and resulted in 37-58% of total volatile solids and 1.15 g COD per g protein (Massé & Masse, 2000). Prandi et al. (2019) reported for the first time, the complete protein characterisation of the different food waste streams (Prandi et al., 2019). Cereal wastes were found to have 9-30% of crude protein on dry basis, oil processing wastes and dairy wastes were reported to be 8-53% and 13-42% (dry matter basis) respectively and fruit wastes and vegetable wastes also showed protein content up to 20%. Some streams were reported to be highly protein rich and of high nutritional value (rich in amino acids) which gives an indication and possibility of real use of proteins from such waste streams in animal nutrition, their synergies with other constituents of the feed, or in other technical applications (Prandi et al., 2019). Several industrial waste streams have been recognised as encouraging source of proteins but still a detailed and comparative characterisation of nitrogen content of the different waste streams, concentration of proteins, protein composition, amino acids and protein integrity is lacking.

4.2 PHA and proteins

In addition to the non-specific cytoplasmic or other cellular proteins in the microbial cell of a PHA producer, some specific proteins as boundary structures on the PHA cellular inclusions are also present. Intracellular PHA granules are surrounded by phospholipid membranes with attached proteins, composed of polyester synthase, intracellular depolymerse, PHA-specific regulator proteins, phasin proteins, and other additional

proteins with unknown functions (Rehm, 2006). In various species, non-enzymatic proteins associated with inclusion boundary known as Phasins that have no apparent enzymatic activity but play structural role by non-covalently attaching to the polyester granules core. Enzymatic proteins i.e. PHA polymerase and intracellular depolymerase inclusions are found to be integral components of these inclusions (Rehm, 2006).

The proteins have similar functions among different microbial species and genera, however, the polymer inclusions displayed characteristic protein boundary with precise pattern of surface arrays that differed among different genera (Stuart et al., 1998). Various microbial genera have displayed their PHA polymer inclusions with a characteristic component of protein boundary that results in a subcellular component in the cytoplasm. These boundary proteins have a variety of lattice like patterns and different amino acid compositions therefore, exhibiting significant diversity in the proteins involved maintaining the characteristic for a genera or a species. All of the non-covalently attached proteins may not be essential for PHA granule formation but plays several biological functions in PHA granule structure, biosynthesis gene regulation and PHA mobilisation (Rehm, 2006).

4.3 Pre-treatment for simultaneous release of PHA and protein

The main steps for concomitant protein recovery with PHA production start with cell disruption or pre-treatment methods, then exposing to filtration or centrifugation to separate the PHA from protein solution, and lastly the precipitation of protein from protein solution or the liquid stream generated (Xiao & Zhou, 2019). Various methods of pre-treatment of the fermented broth containing PHA accumulated cells have been investigated (Kosseva & Rusbandi, 2018). These procedures weaken the firmness of cell wall and the

envelope which subsequently make further steps to solubilize and recover the PHA granules easier and efficient. Moreover, to obtain a higher purity, a purification step after extraction of PHA can be later added (Madkour et al., 2013). Because the main priority is PHA recovery in the process and then comes the protein recovery from liquid streams, the pre-treatment methods have to be selected in accordance to the maximum PHA recovery.

Choosing the adequate pre-treatment methods depends on several factors such as production strain, required purity of product (PHA), impact on molecular mass (acceptable) and availability of the isolation agents. Pre-treatment methods such as heat, alkali treatment, salt treatments or freezing/thawing cycles have been widely reported (Madkour et al., 2013). Heat treatments, alkali treatments or their combinations have been broadly used in PHA recovery which also leads to the release of intracellular components including proteins and their better solubility in the solution (Lorenzo-Hernando et al., 2019). It has been reported that the pre-treatment of *Alcaligenes latus* with NaOH reduced significantly the number of passes in continuous flow bead mill to release most of the proteins (indicators for released compounds) (Madkour et al., 2013).

Alkali-heat treatment is one of the widely used chemical methods due to advantages like simple devices, easy operation and high efficiency. It induces solubilisation of membrane proteins, cell disruption or damage to microbial cell as well as saponification of the membrane lipids (Xiao et al., 2017). Yu et al. (2014) showed high protein solubilisation efficiency in electrochemical treatment, followed by thermal-alkaline treatment, then thermal and lastly in alkaline treatment (Yu et al., 2014). Whereas, Chisti et al. (1992) reported alkaline treatment as the best option for protein solubilisation and recovery from sludge (Chisti, 1998). To our best knowledge, the previous studies based on protein

solubilisation and precipitation have not been specifically addressed in relation to PHA that is, correlation between solubilised protein (content, yield, composition, molecular weight) and PHA content and composition. Also, different pre-treatment methods may affect the related protein molecules weight and type. This could further affect the amino acid composition thus, impacting its application as feed stuff or contact surface and interacting groups in case of proteins used as wood adhesives (Chisti, 1998).

Once the intracellular components (PHA and proteins) are released, the soluble protein and the PHA granules can be easily separated using centrifugation, sedimentation or filtration. From here, the precipitate containing the PHA granules is extracted by a diversity of processes detailed of which have been reported elsewhere (Madkour et al., 2013; Mannina et al., 2020) and the soluble protein solution could be further precipitated for protein recovery (Figure 2).

#Insert Figure 2 here#

4.4 Protein precipitation methods

The methods used for the precipitation of proteins aim to modify either the properties of the protein or the solvent by changing ionic strength, pH, using organic solvents, polymers or their combination. The methods should be evaluated not only in terms of economical and technical feasibility but also take account the waste stream quality, the treatment steps and the final characteristics. For example, for leather tanning industrial wastewaters, various techniques gave different protein removal efficiencies. Around 50% protein precipitation was obtained with isoelectric precipitation (pH 2.1-3.8), 60% protein removal using $FeCl_3$, polyelectrolyte does not gave satisfactory protein precipitation whereas precipitation by magnesium ammonium phosphate and followed by acid resulted in 50%

protein along with 85% ammonia removal (Kabdaşli et al., 2003). Numerous studies have been done which deals with protein precipitation and the most frequently used are described as follows:

4.4.1 Isoelectric precipitation

Isoelectric precipitation is most widely used method for protein recovery from industrial wastewaters. Reduced solubility of proteins has been observed at pH at low ionic strength to the point where protein has a zero net charge (isoelectric point or pI). This effect has been used for proteins of low hydration constants or hydrophobic proteins (Bell et al., 1983). The major disadvantage of this method is the use of acids for obtaining pI that can damage the proteins irreversibly. The protein is sensitive at low pH but this may be greatly amplified due to the use of acid anions from Hofmeister series that are associated with protein destabilisation (Bell et al., 1983).

Alkali solubilisation followed by isoelectric precipitation is commonly employed method for protein recovery from low valuable materials. The method is efficient, gives maximum recovery, and functional and stable proteins devoid of lipids. Marsal et al. (2010) used precipitation of solubilised proteins at their pI because of its technical and economical viability for organic nitrogen removal from wastewaters of tannery beamhouse operations (Marsal et al., 2010). The precipitation of proteins resulted in a decrease of 80-85% COD with protein precipitation of around 68-78% at pH 3.8-3.9. This will not only be beneficial environmentally but also to tanners to save the cost on wastewater treatment and associated taxes. The characterisation showed that the recovered protein meets all the requirements to be used as organic fertilisers. For tanning industry wastewaters, isoelectric precipitation reported 50% protein recovery between the pH 2.1-3.8 (Kabdaşli et al., 2003). Proteins

from the slaughterhouse wastewaters have shown the lowest solubility of protein at pH 4.5. The obtained proteins were of high-quality and could be used in animal feed formulations (Bethi et al., 2020). In addition to pH shift, heat treatment can further enhance the precipitation. For example, a pH shift from 7 to 5 yielded 63% of the protein in surimi wash-water and the subsequent heat treatment (60 ºC) precipitated rest of the proteins in the wash water (Iwashita et al., 2016).

4.4.2 Precipitation using organic solvents

Organic solvents such as ethanol and acetone have been used extensively to precipitate proteins from low ionic strength solutions. Numerous parameters such as solvent concentration, pH, temperature, ionic strength of solution and protein concentration have to be taken in account. On addition of solvent solution containing proteins, the solvating power of water for a hydrophilic charged molecule is decreased as the concentration of organic solvent increases. It can be described in terms of decrease of dielectric constant of solvent or bulk displacement of water and partial immobilisation of water molecules by hydration of organic solvent. Aggregation occurs because of the electrostatic and van der Waal forces. Moreover, size of molecules also effect the precipitation order i.e. large molecules aggregate sooner because of the greater chance of having charged surface area matching with other protein (Bell et al., 1983). Compared to other precipitation methods, organic solvent precipitation gives a better separation, higher degree of selection, easy continuous operation, low energy consumption and large production capacity (Chen & Wang, 2016). This method is widely used at small scale extractions however, due to the requirement of high volumes of solvent at large scale that may be economically and environmentally unfeasible, it is not commonly used for industrial wastewaters.

4.4.3 Neutral salts precipitation

Salting out is defined as the process when solubility of the non-electrolyte in water reduces with increasing salt concentration (Hyde et al., 2017). When the salt is added to the protein solution, there is an increase in the surface tension of water which causes hydrophobic interaction between protein and water molecule. The protein decreases its surface area to lessen the contact with solvent. It is done by folding and self-association which leads to precipitation of proteins (Wingfield, 2016). Ammonium sulfate being cheap, readily available, highly soluble in water than other phosphate salts, and causing low risk of protein denaturation due to low heat of solubilisation, is the perfect reagent of choice for salting-out. However, during the process, contaminants may get precipitated in the protein fraction. In addition, removal of salts from protein sample may be required that would further necessitate the processing/purification thus, increasing the protein recovery cost (Wingfield, 2016).

Studies have compared different methods such as salting-out using ammonium sulfate, isoelectric precipitation and organic precipitation and revealed that the salt precipitation to be most effective (Xiao et al., 2017; Zhi et al., 2020). Zhi et al. (2020) recovered 75% of the protein using ammonium sulfate along with other value-added substances such as coenzyme Q_{10}, carotenoids, bacteriaochlorophyll and 5-Aminolevulinic acid from photosynthetic bacteria treating wastewater (Zhi et al., 2020).

4.4.4 Charged polyelectrolytes (PE)

Ionic polysaccharides have been extensively used in precipitation of food proteins. The complexation of protein and PE is driven by combination of electrostatic interactions and entropy gains when the counter-ions in the proximity of protein and PE chains are released

(Gao et al., 2019). Initially, the addition of PE to the protein solution produces a liquid-liquid phase separation that denotes destabilisation. When PE concentration is increased, the precipitation is enhanced however, increasing the PE above optimal value will cause the aggregated colloid to redissolve causing the state of restabilisation. The concentration of PE for stabilisation or destabilisation depends on pH of the protein solution, as well as on ionic strength and molecular weight of PE (Jiang & Prausnitz, 1999). Use of oppositely charged PE can lead to high protein recoveries with small amount of PE while retaining the native state of protein without any biological activity loss and further, the PE could be recycled.

Mostly studies have reported the use of acidic polysaccharides such as carboxymethylcellulose (CMC), polyacrylic acid (PAA), polymethacrylic acid (PMA), alginate, carrageenan and pectate for protein precipitation. Polyethyleneimines are widely used as flocculating agents in wastewaters for proteins and enzymes purification. Polyethyleneimines complex with anionic substances is strongly affected by the types and concentration of salt anions present and pH (Bell et al., 1983). Hydroxyethylcellulose, lignosulphonate, xanthum gum, and chitosan have been used to precipitate proteins and fats from food processing industries wastewaters. CMC is generally recognised as safe (GRAS) and could be used as supplements in animal feed. Yadav et al. (2014) evaluated precipitation of soluble proteins from yeast-cultivated cheese whey using CMC and thermal treatment followed by CMC precipitation (Yadav et al., 2014). The latter process gave total soluble protein recovery of 81% from the supernatant without centrifugation (Table 2). Kurup et al. (2019) reported recovery of 46% of proteins using Na-lignosulphonate from dairy wastewater (Kurup et al., 2019a). It has also been reported that

lignosulphonate at 1% concentration has effectiveness of 3% bentonite which makes its use more cost-effective. The advantages of organic polymers such as CMC and lignosulphonate is that they are effective at low concentrations and they reduce organic load in treated water. Several factors such as ionic strength or pH of the solution, charge density, molecular weight and dosage of the polyelectrolyte as well as manner of polymer addition have been found to affect the protein removal and fractionation efficiencies using these polymers (Clark & Glatz, 1987).

4.4.5 Non-ionic polymers

Among polymers, most studied are polyethylene glycols and dextrans. They exclude the proteins from the solution and reduce the amount of water available for their solvation. Aqueous two-phase separation (ATPS) in polyethylene glycol (PEG) and potassium phosphate mixtures has been commonly used for protein recovery from biological extracts and fermented broths. ATPS has been known to remain stable in performance with different feedstocks and process fluctuations. However, operational changes of cell disruption processes or different methods of cell disruption may impact the performance of protein fractionation in following stages of downstream processing. Also, reuse of the phase-forming chemicals is an economic option but can affect process stability by recycling solutes after certain number of cycles (Rito-Palomares & Lyddiatt, 1996). ATPSs containing PEG 10000 and biodegradable citrate salts i.e. sodium citrate, potassium citrate and ammonium citrate were developed for recovery of proteins from tannery wastewater systems. During the partitioning, the system containing PEG 10000 and sodium citrate was superior than other systems used and recovered 95.85% of proteins from tannery wastewater in bottom phase (Raja & Murty, 2012).

4.4.6 Polyvalent metal ions

Polyvalent metal ions can be categorised into 3 groups i.e. ions strongly binding to carboxylic acids and nitrogenous compounds (amines, heterocyclics) such as Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+}; strongly binding to carboxylic acids but not to nitrogenous compounds such as Ca^{2+}, Ba^{2+}, Pb^{2+}, Mg^{2+}; and strongly binding to sulphydryl groups such as Ag^{+}, Hg^{2+}, Pb^{2+}. Metal ions employed in low concentrations for precipitation of proteins can be subsequently removed by chelating agents or ion exchange resins (Bell et al., 1983). $CaCl_2$ and $FeCl_3$ is found to be effective coagulants to recover proteins from dairy effluents. Around 80% of the protein was recovered from dairy wastewaters using 0.07% (w/v) $FeCl_3$ at pH 10. When the pH was decreased from 10 to 4, the recovery percent reduced to 54% that depicted the lack of $FeCl_3$ and protein component interaction at lower pH due to positive charge on them which lowered the electrostatic attraction and flocs formation (Kurup et al., 2019b).

Xu et al. (2001) performed the economic analysis of 4 coagulants (lignosulfonate, CMC, bentonite, $FeCl_3$) to treat egg processing industrial wastewater. The total savings of each coagulant exceeded USD $22000/million gallons (MG) (Xu et al., 2001). Largest cost savings of $24000/MG were obtained for lignosulfonate and $FeCl_3$. Based on the wastewater volumes (around 42.5/yr), an annual savings of around $1 millions could be done using precipitation or coagulation technique. The precipitation methods have advantages such as low cost, high yield, simple operation, high efficiency and therefore, the precipitation method is widely used in downstream processing for proteins. Among the methods described, isoelectric point precipitation and polyelectrolyte precipitation are the methods with greatest potential for industrial wastewater treatment applications. It is

significant to understand and compare the efficiencies of all the precipitation methods. Combinations of the conventional precipitation techniques as well as new sustainable technologies are required for fractionating, and concentrating the protein from liquid waste streams. In addition, comparison on economic implications and the overall recovery cost is not very clear and further research in this area is required.

4.4.7 Membrane separation

Membrane filtration processes have been used in the processing of dairy products, beverages, oil, meat, fruits and vegetables, and sugar. Several studies have been conducted using ultrafiltration for removal of total solids and COD in the wastewater. Combinations of ultrafiltration and dehydration can produce by-product containing 30-35% protein and 24-45% fat from poultry wastewater having the amino acid composition comparable to soybean meal. The cost estimation of recovered protein based on 60% recovery gave approximately USD$ 24000 per day from a plant processing 100,000 chickens per day. This monetary value can claim the operating cost and certain part of facility investment thus, maintaining the economy of the process, making it competitive to the existing systems. Lo et al. (2005) recycled the poultry wastewater by ultrafiltration (Lo et al., 2005). All crude proteins in the poultry wastewater were recovered using a polysulfone membrane of molecular-weight-cut-off of 30 kDa reducing the COD by 58.86%. They found that severe fouling of membrane was inevitable however, flushing the ultrafiltration membrane with cleaning reagent (sodium hypochlorite) after processing was capable of restoring membrane performance. Das et al. (2016) used ultrafiltration to separate protein and lactose with high purity and yield (80% and 90% respectively) from whey. Ultrafiltration use for protein recovery (sericin) and recycling of process water has been performed in the silk

manufacturing degumming process (Fabiani et al., 1996). Membrane filtration have more advantages than other techniques as it can separate protein from other impurities. Also, it offers concentrating protein which can decrease the protein loss in post-precipitation step. However, this technique is comparatively expensive and depending on the applications, one or more techniques maybe required in combination (Aramwit et al., 2012). Microfiltration can be used to pre-treat the wastewater before ultrafiltration to remove suspended particles to decrease the fouling of ultrafiltration membrane.

#Insert Table 2 here#

4.5 Characterisation of protein and its functional properties

Based on the amino acid composition, solubility in various solvents, molecular weight, geometrical conformation, sedimentation behaviour, surface charge distribution or ability to maintain their native molecular configuration, proteins can be characterised and grouped. They have both bio-functional and techno-functional properties. Bio-functional properties mostly refer to nutritious properties for their application in as feed/food and in pharmaceutical sectors. Techno-functional properties relate to building up the structures technical applications like packaging with properties like solubility, network formation and viscosity being the most relevant (Coltelli et al., 2016). Protein solubility is one of the pre-requisites for stable protein dispersion in coating layers and for increasing the viscosity and network formation. The supernatant left after the protein precipitation can also be characterised for minerals and other nutrients. In that case, it could be used for biomass growth and PHA production in subsequent fermentation rather than releasing it for treatment (Figure 2).

4.6 Case studies of protein recovery

Cahú et al. (2012) presented an interesting process to increase the sustainability of shrimp processing wastes with respect to circular bioeconomy approach. They developed a process for recovering bioactive molecules such as protein hydrolysates, carotenoids, chitin, chitosan, glycosaminoglycans from white shrimp *Litopenaeus vannamei* processing waste and side streams during the recovery process (Cahú et al., 2012). The process was carried out on a pilot scale and can be used up to the volumes of 1000 L. The shrimp waste was thermally inactivated and enzymatic autolysis and centrifugation was carried out. The liquid portion was centrifuged again to obtain protein hydrolysate in the supernatant and carotenoprotein in the precipitate. Chitin was attained after the washed carapace (solid phase) was demineralized, deproteinized and bleached. Alkaline deacetylation of chitin produced chitosan. Moreover, calcium and the protein concentrate from chitin purification was also obtained. One kg of shrimp processing waste resulted in 48.1 g protein and calcium concentrate, 44 g of an ethanolic extract (i.e. lipid concentrate), 53 g of carapace content and 1.5 L of protein hydrolysate.

Bourtroom et al. (2008) recovered proteins from surimi wash water by changing the pH and using organic solvent (Bourtoom et al., 2009). Maximum precipitation (66.6 g protein/100 mL wash water) was obtained at pH 3.5. Increasing the organic solvent resulted in higher protein precipitation with highest precipitation (i.e. 65 g protein/100 mL wash water) using ethanol concentration of 60g/100g wash water. They showed with the optimum conditions precipitation of proteins form surimi wash water can be enhanced.

Torres et al. (2007) showed five steps to recover fish proteins from waste streams, 1) homogenisation of by-products to reduce the particle size and increase surface area for protein solubilisation, 2) solubilisation of fish proteins at acidic or basic pH, 3)

centrifugation to separate the solution into fractions (light, medium and heavy) of fish oil, solubilised muscle protein and fat-free impurities, 4) precipitation of proteins from medium fractions by changing the pH to pI i.e. 5.5 of fish muscle proteins and 5) recovery of precipitated proteins by centrifugation that could be used as food ingredient (Torres et al., 2007).

Whey being a major by-product of dairy industry contains whey protein 6-10 g L^{-1}(sweet whey), 6-8 g L^{-1} (acid whey), BOD ranging from 30-50 g L^{-1} and COD around 60-80 g L^{-1} thus, making the disposal difficult for dairy industry (Das et al., 2016). Two-stage foam separation technology has also been used for recovering proteins from whey wastewater. The effects of inclined angle, volumetric air flow rate, temperature, pH on performance was investigated. The total protein recovery that was obtained using this technique was around 80%. Foam separation method requires low investment, simple equipment, environmental compatibility and low energy consumption (Jiang et al., 2011).

Cereal side streams serves as potential streams for protein sources. The global annual production of rice and wheat are 27 and 167 million tons respectively, out of which 15% is protein (Sozer et al., 2017). Milling and starch, bioethanol production can produce high amounts of protein-rich wheat and rice side streams that can be utilised as feed. Proteins extraction from cereal side streams are challenging as the proteins are trapped within the cell wall matrix of cereals. Van den Borne et al. (2012) showed the disintegration of wheat bran using acidic treatment that improved the protein digestibility in vitro and did not affect the quality of protein for animal feeding (van den Borne et al., 2012). Various other conditions of hydrothermal treatment using high temperatures and acidic pH resulted in high yields of proteins. Other than these, enzymatic processes and thermomechanical

methods are also used for a better extractability of proteins. It has been observed that when the recovery of proteins using alkaline extraction is low, enzymes such as carbohydrolases, peptidases and phospholipase under neutral or mild acidic or alkaline conditions could enhance the solubility of proteins. The enzyme used and the conditions applied for its use like pH, solid-liquid ratio, temperature, extraction time, and extraction cycles needs to be evaluated for maximum recovery. However, the cost of the enzyme is a critical factor to assess the economic feasibility of the process and therefore, this process is generally not used at large scale (del Mar Contreras et al., 2019).

5. Proteins as feed, food and further

5.1 Feed market

Protein consumption for animal feed is predicted to increase by more than 50% by the year 2030 when compared to 2000 (Prandi et al., 2019). The interest in using waste streams for animal feed is growing quite popular with various East Asian regions working on food waste recycling on the basis of strict regulations and rendering food wastes through several treatments (Salemdeeb et al., 2017). In South Korea, as of 2010, there were 259 manufacturers of protein feed, producing pig feed from food wastes. Salemdeep et al. (2017) showed that recycling of municipal food waste as pig feed is better in terms of environmental and health impacts as compared to anaerobic digestion and composting (Salemdeeb et al., 2017). Hwang et al. (2008) showed the opportunity of using proteins as animal feed which were recovered from sludge (Hwang et al., 2008). The sludge was pre-treated using alkali treatment followed by ultra-sonication to release proteins. The crude proteins were recovered by precipitation at isoelectric point followed by drying. Nutrient compositions were similar to conventional protein feeds and heavy metals were absent in the recovered proteins.

Other than pigs, protein feed from wastes can be used for feeding poultry, fish and ruminants. Several food wastes discharged from industries during processing (tofu waste, bread waste, waste cooking oil, soy sauce waste) have been fed to fish species farmed in China such as Chinese carp, and Tilapia which grew well on the waste-based diets showing suitability of such waste streams to make fish feed (Mo et al., 2018). Mo et al. (2014) showed that food waste based diets contained suitable levels of essential amino acids, proteins, crude lipids and carbohydrates suitable for feeding low-trophic level fishes (Mo et al., 2014). The market values of proteinaceous nitrogen from vegetable sources or food streams can be obtained at current price of USD$ 1.24-1.6. Yet, there is so far no clear data of bacterial based protein products from waste streams in terms of their putative market size.

5.2 Adhesives

Bio-based feedstock use for wood adhesives have gained popularity because of the environmental and climate change issues associated with synthetic adhesives. For example, formaldehyde based resins, emit volatile organic compounds and are carcinogenic in nature. Protein-based adhesives derived from food crops (for example, soybean) have been very common however, alternative feedstocks are required which do not compete with human and animal food resources.

Pervaiz and Sain (2011) used sludge (kraft paper mill secondary sludge) to recover protein and investigated it for wood adhesive (Pervaiz & Sain, 2011). The process involved floc structure disruption of sludge in alkaline medium followed by soluble protein recovery from aqueous phase by low pH precipitation. The recovered protein contained low quantity of lipids and ash than raw sludge which are reported to be unfavourable for adhesion

properties and contained lignin and carbohydrates that are considered as adhesion extenders and used it for formulations in wood adhesives to improve adhesion properties. Whey protein from cheese industrial wastes has been blended with sucrose and used for formulating adhesives in paper industries (Wang & Guo, 2014). The whey protein-sucrose based glue had good bonding strength and can be applied to different substrates like paper-paper, paper-wood, paper-metal, and paper-plastic. These protein-based adhesives can be easily washed away from paper fibres during recycling and pulping process in contrast to the traditional gluing material that stick onto the fibres thus, making them less suitable for new paper products (Coltelli et al., 2016). The improvement in the adhesion properties of isolated proteins could be further improved by using hybrid formulations of these proteins with other high strength adhesives.

5.3 Bio-based plastics, films and coatings

Lately, the focus has shifted to the second generation of bio-based polymers production from materials from the inedible or waste streams or low-value by-products from industries (rendering, food processing, and agricultural sectors). Animal protein streams such as chicken feathers meal and, meat and bone meal have been successfully converted and transformed into protein-based plastics and thermosetting materials. Soy dreg (SD) is a by-product in the isolation process of soy protein and is used as a raw material for making biodegradable plastics. Water resistant plastics have been built using SD as compatibilizer to exhibit a better biodegradability and water resistance.

Unfolding of proteins and its dissociation into subunits when treated with heat, acid/base and solvents makes them loose their native conformation and show free reactive group that were initially bonded. This step is required to generate new intermolecular bonds between

the protein molecules and thus forming networks or films through hydrogen, hydrophobic, ionic, and covalent bonding (Coltelli et al., 2016). The protein hydrophilicity promotes compatibility with polar surfaces such as paper and provide barrier to apolar gases like carbon dioxide and oxygen making them suitable for coatings and films. The unique structures of proteins confer them various functional properties like high intermolecular binding potential which allows protein-based films to perform better than fat and polysaccharide-based films. Proteins could be used as coatings on materials in packaging industry where they could provide properties such as water and gas barrier and sealability. For example, whey protein coatings when applied on paper have shown to be good barriers by improving oil resistance and decreasing water vapor permeability and exhibited good visual and high mechanical properties (Coltelli et al., 2016).

5.4 Flocculants and surfactants

Another emerging application of microbial or animal waste proteins is as feedstock for the production of coagulants and flocculants to be used in wastewater treatment. Due to their high-solubility in aqueous solution and polyampholyte nature, proteins can be used as feedstock for flocculants. The flocculation activity of proteins could occur through bridging and electrostatic interaction mechanisms. Modification of protein is required to enhance the flocculation ability which can be done via changing the pH of solution, capping certain functional groups in proteins or grafting protein with other polymers. For example, methylated soy protein flocculants have been developed and showed higher flocculation performance with kaolin clay and loam as compared to chitosan (Mekonnen et al., 2016).

Surfactants have universal molecular structure containing hydrophilic and hydrophobic groups with property of absorption at interfaces, foaming properties, reducing interfacial

tension and emulsification. Proteins being amphiphilic molecules also exhibit surface properties and due to their biodegradability and low-toxicity, they have been used as surfactants. However, several modifications such as phosphorylation, acylation, succinylation and acetylation have to be performed to improve their stability and foaming ability (Mekonnen et al., 2016).

6. Recycling of the supernatant or waste stream generated in PHA process

After the fermentation and treatment of fermented broth, the supernatant obtained after separating the PHA granules will contain various nutrients (Koller, 2015). Therefore, this supernatant or waste stream generated rather than using for protein extraction, can be utilised as a nutrient feed stock for PHA production. The importance of recycling the waste stream in the process is that it will not only make the process more economical because of the requirement of less chemicals in production but also diminish the ecological risks by reducing the load of wastewater on WWTP treating it. Recycling the waste streams generated in the process for the growth of PHA-producing microbes is a classic example of circular bioeconomy to maintain circularity.

Case study of high saline side streams recycling produced using Haloferax mediterrannei:

Koller (2015) investigated recycling of waste streams of the PHA production process in a very interesting way. He used halophile *Haloferax mediterranei* for PHA production using whey as substrate (Koller, 2015). *H. mediterranei* has some excellent properties such as broad spectrum of substrate, genetic stability, produces good quality of PHA, and marketable side products such as bacterioruberins or extracellular polymeric substances (EPS) which makes it an attractive microbe for the production of PHA. This extremophile adaptation to high salinity offers the opportunity to run the process at low operation costs

as low energy will be required because of no sterilisation and no solvents are needed for product recovery due to cells higher inner osmotic pressure. Using whey gives the opportunity to use carbon-rich industrial waste streams as feed stock for PHA. Cost estimation reports the PHA production at price below USD$ 3.29 using *H. mediterranei* on whey lactose.

In the experiment, PHA-rich cells were separated from the aqueous phase of fermented medium from where PHA is isolated from the biomass by using cell disruption in hypotonic medium (water). PHA granules and cell debris were separated using centrifuge, enabling PHA granules separation by simple skimming. As a result, 3 different waste streams from the entire process were released: 1. Supernatant of the fermentation broth (SSF), 2. Cell debris along with intracellular salts after PHA recovery (saline fraction or SF), 3. Salt-free cell debris obtained when the saline debris was washed after disruption and PHA separation (Figure 3). It was observed that SSF can act as a saline media for the subsequent cultivation of cells (fed-batch fermentation) however, as compared to the fresh medium, lower productivities and growth rates were observed. PHA concentration and protein concentration decreased from 7.2 and 6 g/L in original fermentation to 2.28 and 0.94 g/L in recycling fermentation respectively. The SF solid material was produced by removing water using heat treatment. This solid fraction was utilised as a substitute of salt in subsequent cultivations. The results showed that the saline fraction is an important source of nitrogen, phosphate and carbon and can be used to replace amounts of NaCl. However, the cells cultivation is possible to a certain extent due to the negative impacts on cell growth at different amounts of supplementation. The use of salt free cell debris or SD as a substitute for yeast extract was also investigated. It was observed that only small amounts of the cell

debris were converted into new biomass. An additional step of hydrolysis of cell debris to make it accessible to the cells can be done or may be supplemented by certain vitamins present in yeast extract but not in cell debris.

#Insert Figure 3 here#

7. Challenges and perspectives

It is imperative to maintain a balance between product yields, processing cost, and energy consumption to ensure and boost the efficiency and profitability to be achieved by the commercial production of protein. The greatest challenges in creating and maintaining circular economy in a process is to study the integration of technologies, and expansion of prospects to integrate the biological systems and/or bioprocesses across the waste management models and systems (Mohan et al., 2016). One major challenge observed in the process is that the lack of operational/environmental standards and incentive policies for value-added products recovery from wastewaters that may hinder the shift of these bio-products recovery from the laboratory-scale study to the commercial market (Xiao & Zhou, 2019). Protein recovery and/or liquid stream recycling is a new domain that will integrate circular bioeconomy in the PHA process, however, it has not been applied to real life and requires further research and strong collaboration of researchers, industrialists and environmentalists. Future industrial developments concentrated on the valorisation of industrial waste streams ought to be based on the available quantities of liquid streams, their characterisation, analysis of techno-economic potential and life-cycle based environmental assessment of merits and demerits. Moreover, the precipitated proteins may contain heavy metals when precipitated from industrial wastewaters depending on the

characteristics of waste streams and protein precipitation method used. Therefore, standard testing depending on the application niche of the recovered protein have to be developed. New, feasible and sustainable technologies are required at different processing stages starting from fractionating, concentrating and isolating the protein from solid or liquid side streams, then for improving the technological functions and sensory properties of protein fractions depending on the applications of performance proteins or dietary proteins and lastly, assessing the process in terms of sustainability and socioeconomic performance of the product system. Although further studies are essential to achieve protein recovery and purification with respect to its applications, a new perspective in liquid streams management during PHA production or other industrial side streams is open by recovering the valuable compounds present.

8. Conclusions

Sustainability in PHA process must be established on the standards of 'Zero Waste' and 'Cleaner Production'. Side streams of PHA contains high nutritional value that can be utilised in sustainable fashion. Obtaining microbial proteins and recycling the liquid streams in the process is a complementary approach in biorefinery. Reaching the goal will depend on choice of feedstock, characteristics of liquid stream, optimisation of the extraction procedure and collocation of the recovery processes in production chain of PHA. The cascading processes used should be cost-effective and the mechanism behind protein recovery should be further established with their applications in industry.

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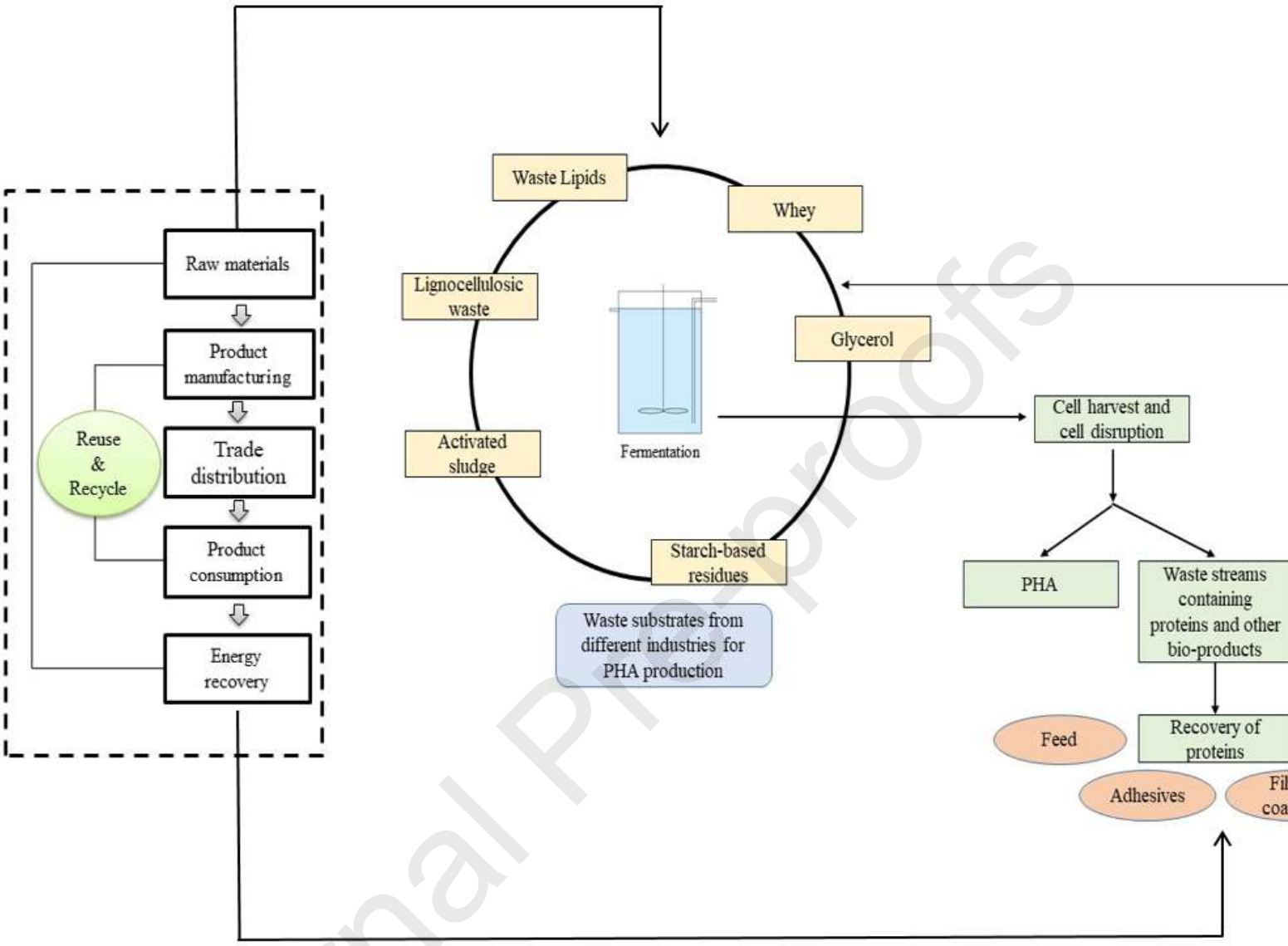


Figure 1. Circular bioeconomy approach in PHA production

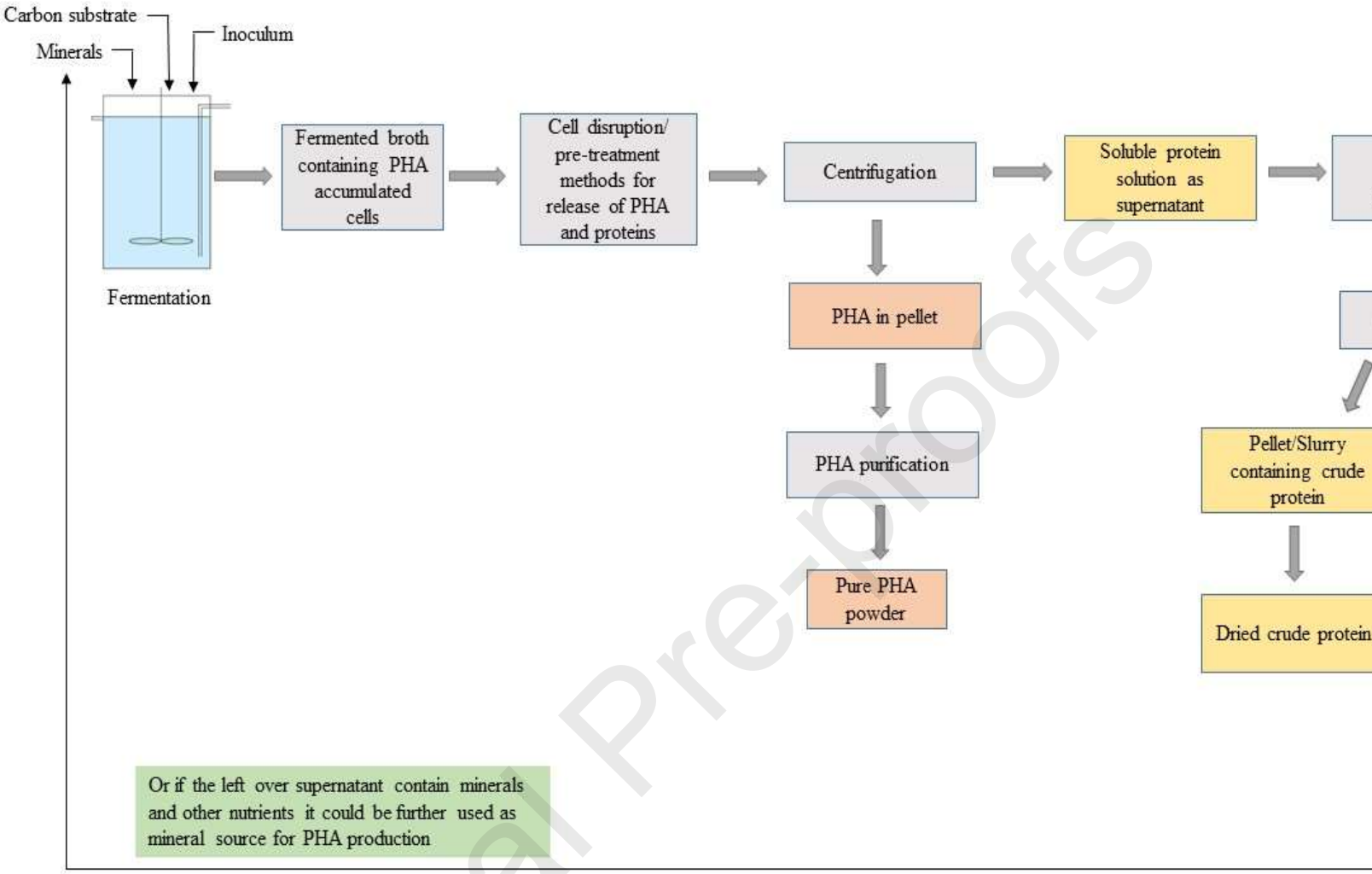


Figure 2. Simultaneous recovery of PHA and microbial proteins during PHA production process

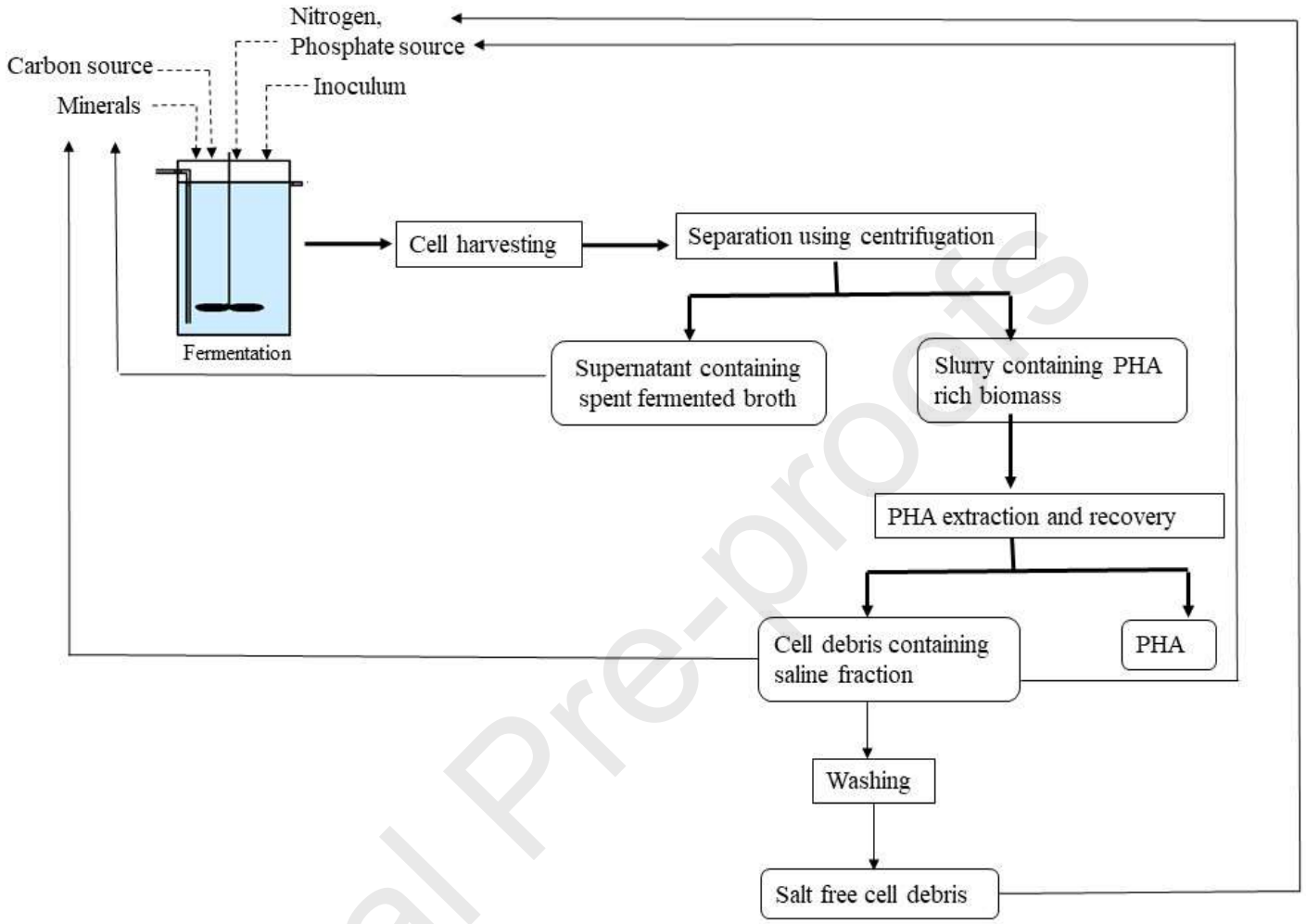


Figure 3. Diagrammatic representation of recycling during PHA production using *H. mediterranei*

Table 1. Overview of PHA production by different strains and their concentration

Microbial strain	Type of PHA	Operation mode	PHA concentration (g l^{-1})	PHA cont
Substrate- Starch and sucrose-based materials				
Azotobacter chroococcum 23	P(3HB)	Fed-batch	25	46
Bacillus cereus CFR06	P(3HB)	Batch	0.48	48
Cupriavidus necator NCIMB 11599	P(3HB)	Fed-batch	51.1	70
Cupriavidus sp. KKU38	PHA	Batch	2.8	65.3
Recombinant E. coli SKB99	P(3HB)	Batch	1.2	40
Recombinant *E. coli* (HMS174/ pTZ18u-PHB) (*C. necator* genes)	P(3HB)	Fed-batch	31.6	80
Pseudomonas fluorescens A2a5	P(3HB)	Batch	22.4	70
Substrate- Lignocellulosic materials				
Burkholderia sacchari IPT 101	P(3HB)	Batch	2.73	62
Burkholderia cepacia IPT 048	P(3HB)	Batch	2.33	53
Recombinant E. coli (*C. necator* genes)	P(3HB)	Batch	1.7	35.8
Ralstonia eutropha ATCC 17699 (*C. necator*)	P(3HB)	Batch	11.4	75.5
Substrate- Whey-based culture media				
Recombinant *E. coli* (*C. necator* genes)	P(3HB)	Batch	5.2	81.3
Recombinant *E. coli* (*C. necator* genes) GCSC 6576	P(3HB)	Fed-batch with oxygen limitation	25	80
Pseudomonas hydrogenovora DSM 1749	P(3HB) and P(3HB-*co*-3HV)	Fed-batch without oxygen limitation	32	57
Recombinant *E. coli* K24 K (*Azotobacter sp*. genes)	P(3HB)	Fed-batch	1.27	12
Alcaligenes latus	PHB	Batch culture	0.11	n.r.
Engineered *Escherichia coli* with *Alcaligenes latus* PHA biosynthesis genes	PHB	Fed-batch culture	2.57, 4.6, 1.35	n.r.
Substrate- Solid agro-industrial by-product				
Recombinant *Bacillus subtilis* 1A304 (105 MU331)	P(3HB)	Batch	0.06	2.5

Azotobacter vinelandii UWD (ATCC 53799)	P(3HB-*co*-3HV)	Batch	0.43	37
Substrate- Sludge from cardboard industry				
Enterococcus sp. NAP11	PHB	n.r	5.236	79.27
Brevundimonas sp. NAC1	PHB	n.r	4.042	77.63
Substrate- Oils and waste lipids				
Pseudomonas aeruginosa NCIB 4004	mcl-PHA	Flask	2.0	66
C. necator H16	3HB	Batch reactor	3.5	57

n.r.- not reported; P(3HB)- Poly(3-hydroxybutyrate); mcl-PHA- medium chain length polyhydroxyalkanoate; P(3HB-*co*-3HV): Poly(3-hy

Table 2. Protein recovery studies from different industrial waste streams

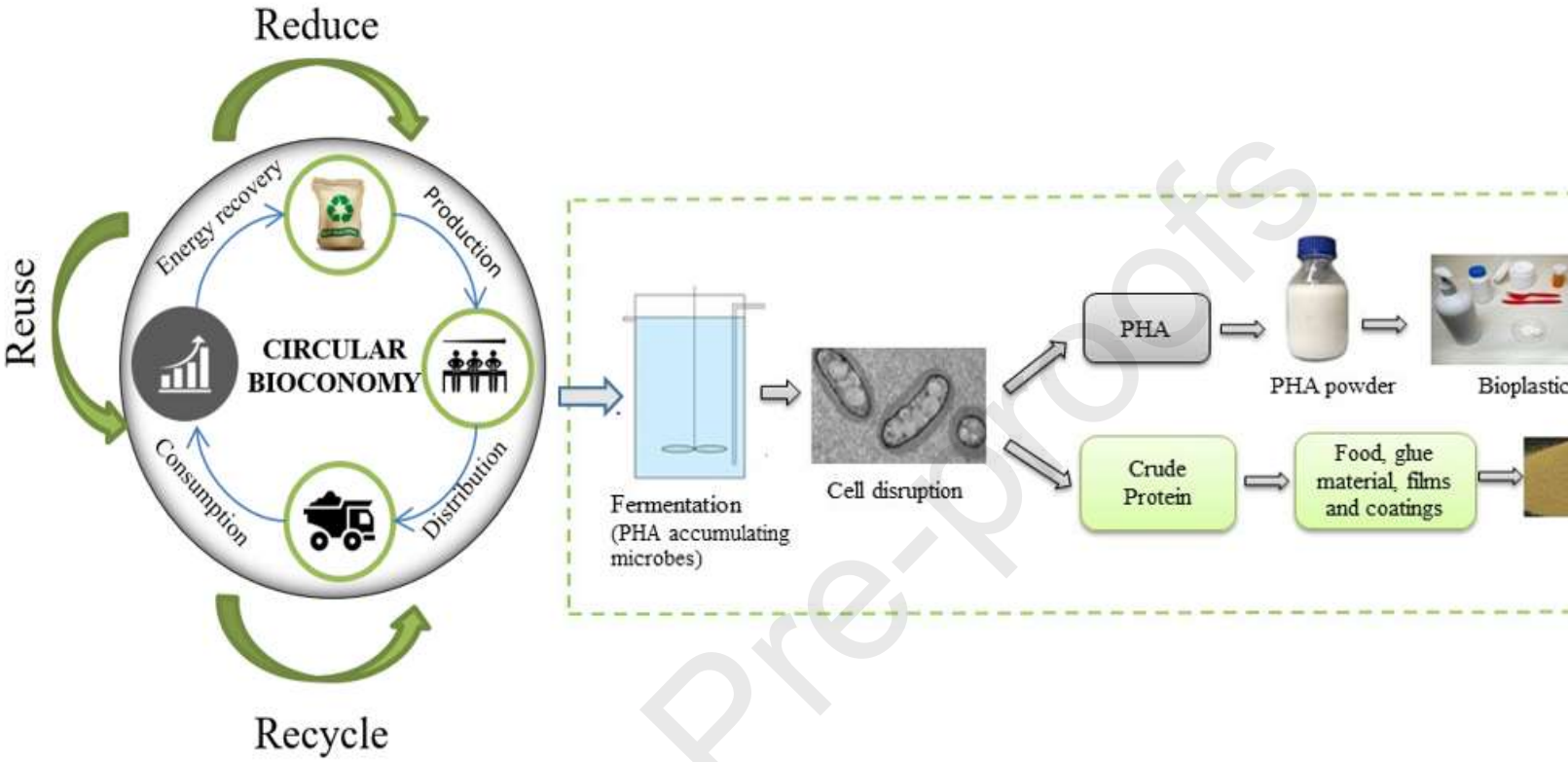
Source	**Methods of recovery**	**Removal/recovery%**	**Characterisation of protein**	**Appl**
Isolated soy proteins wash waters	Ultrafiltration (5, 20, 50 kDa)	Best results using 5 kDa with removal of 52% proteins, 34% COD	8-50 kDa	Use i produ
Dairy wastewater	Ultrafiltration	10 kDa resulted in recovery of 100% lactose and 95% protein	20-25 kDa	-
	Sodium-lignosulphonate (polyelectrolyte)	46% of proteins and 96% lipids	-	-
	Ultrafiltration and nanofiltration	90% lactose and 80% protein recovery	20-25 kDa	Can b
Poultry wastewater	Ultrafiltration	30-35% protein recovery	-	Can b
Whey wastewater	Two-stage foam separation	80% protein recovery	18-25 kDa	-
	Heat-acidic precipitation	68% protein recovery, 62% COD removal		Can b
	Thermal precipitation followed by CMC treatment	81% protein recovery		anim
Cereal waste streams	Wet-alkaline extraction	33-38% rice bran protein concentrates	-	Can b

	High pH and temperatures	92% protein recovery	-	Anim
Surimi wash water	Acidic treatment, organic solvent treatment Isoelectric solubilisation and precipitation	65-66 g protein precipitation/100 mL surimi wash water 78-91% proteins precipitated	23.3-71.6 kDa -	Can b prote emul edibl
Wastewater of tannery beamhouse operations	Precipitation by sulphuric acid at isoelectric pH	68-78% protein precipitation, 80-85% COD removal	-	Can b organ
Leather tanning industry waste water	Isoelectric pH (2.1-3.8) Ferric chloride Magnesium ammonium phosphate followed by acidic treatment	50% protein precipitation 60% protein precipitation 50% protein precipitation with 85% ammonia removal	-	Can b organ
Wastewaters from silk degumming processes	Ultrafiltration (20-30 kDa)	Around 97% protein recovery, 92-98% COD removal	-	Can b cosm feed

Highlights

- Application of circular bioeconomy approach in PHA production process to enhance process market implementation.
- Recovery of microbial proteins released in the liquid streams and/or recycling the streams for PHA production to improve circularity in the process.
- Biorefinery approach for precipitation of proteins from waste streams with their industrial applications.

Graphical abstract



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