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MINI REVIEW

Application of the solid-state fermentation process and its variations in PHA production: a review

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Abstract

Solid-state fermentation (SSF) is a type of fermentation process with potential to use agro-industrial by-products as a carbon source. Nonetheless, there are few studies evaluating SSF compared to submerged fermentation (SmF) to produce polyhydroxyalkanoates (PHAs). Different methodologies are available associating the two processes. In general, the studies employ a 1st step by SSF to hydrolyze the agro-industrial by-products used as a carbon source, and a 2nd step to produce PHA that can be carried out by SmF or SSF. This paper reviewed and compared the different methodologies described in the literature to assess their potential for use in PHA production. The studies evaluated showed that highest PHA yields (86.2% and 82.3%) were achieved by associating SSF and SmF by *Cupriavidus necator*. Meanwhile, in methodologies using only SSF, *Bacillus* produced the highest yields (62% and 56.8%). Since PHA (%) does not necessarily represent a higher production by biomass, the productivity parameter was also compared between studies. We observed that the highest productivity results did not necessarily represent the highest PHA (%). *C. necator* presented the highest PHA yields associating SSF and SmF, however, is not the most suitable microorganism for PHA production by SSF. Concomitant use of *C. necator* and *Bacillus* is suggested for future studies in SSF. Also, it discusses the lack of studies on the association of the two fermentation methodologies, and on the scaling of SSF process for PHA production. In addition to demonstrating the need for standardization of results, for comparison between different methodologies.

Keywords Solid-state fermentation (SSF) · Polyhydroxyalkanoate (PHA) · Poly(3-hydroxybutyrate) [P(3HB)] · Submerged fermentation (SmF) · Biopolymer · PHA production

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Glossary

Free amino nitrogen (FAN)	Amount of free nitrogen, which microorganisms can assimilate, present in the medium. It is released from amino acids and small peptides.
Polyhydroxyalkanoates (PHAs)	A class of biopolymers produced intracellularly by microorganisms as a carbon and energy reserve.
Poly(3-hydroxybutyrate) [P(3HB)]	Biopolymer belonging to the class of PHA's.
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)]	Copolymer belonging to the class of PHA's.
Poli(3-hydroxyvalerate) (P3HV)	Copolymer belonging to the class of PHA's.
Poly(hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV)	Copolymer belonging to the class of PHA's.
Poly-β-hydroxy-2-methyl valerate (PH2MV)	Copolymer belonging to the class of PHA's.
Polyurethane Foam (PUF)	It is a flexible foam made of polyurethane (PU), a plastic polymer widely used for filling purposes, acoustic insulation and in civil construction, acting mainly as a sealant and thermal protector.
Submerged fermentation (SmF)	Fermentation process characterized by having a liquid substrate.
Solid-state fermentation (SSF)	Fermentation process characterized by being carried out in a solid matrix with low water disponibility, containing nutrients, substrate and carbon source.
Solid-state enzymatic hydrolysis (SSEH)	Fermentation process consisting of two steps in solid medium, proposed by Martínez-Avila et al. (2021).

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable and has other valuable traits including thermoplasticity and production from renewable sources (Kumar et al. 2020). These provide carbon and energy for the bacteria that produce PHAs by forming intracellular granules (Alves et al. 2017). They can be applied in drug delivery, tissue engineering (Raza et al. 2018) and the manufacturing of furniture packaging (Anjum et al. 2016).

Poly(3-hydroxybutyrate) [P(3HB)] is the most characterized PHA (Anjum et al. 2016; Alves et al. 2017; Li and Wilkins 2020). However, yields remain low and expensive, reaching costs four to ten times higher than conventional plastics (Kosseva and Rusbandi 2018), hindering its commercialization (Squio and Aragão 2004). The main reasons for such high production costs are the substrates used and the separation processes (Ramsay et al. 1990).

The optimization of techniques is needed to increase productivity and reduce costs, improving their industrial competitiveness. Such techniques include selecting strains with higher intracellular PHA accumulation, use of agro-industrial by-products as a substrate (Rodriguez-Perez et al. 2018), improvement of microbial strains (Lin et al. 2017; Tran et al. 2016) and optimization of fermentation conditions (Alves et al. 2017).

Submerged fermentation (SmF) is the most used fermentation process for PHA production, while solid-state fermentation (SSF) is less common (Sindhu et al. 2015). SmF is characterized by having liquid substrates, high humidity, and rapid consumption of nutrients (Subramaniyam and Vimala 2012), whereas SSF presents a solid matrix with low humidity and/or absence of free water in the medium (Singhania et al. 2009). SSF has various characteristics that can reduce the production costs of PHAs, such as: lower energy expenditure and operating cost, high efficiency, and allowing the use of various agro-industrial by-products as support and/or substrate (Sharma and Bajaj 2016). These characteristics can be seen as advantages that SSF has over SmF.

SSF has been evaluated in literature as an alternative to SmF. However, there have been studies that associated the two processes, and, recently, a study (Martínez-Avila et al. 2021) that evaluated the association of the two steps of SSF. There is still a need to optimize the production of P(3HB). The evaluation of different fermentation conditions is one of the methodologies cited in literature for this purpose (Alves et al. 2017). Therefore, the aim of this study was to review and analyze the different methodologies, comparing these processes and evaluating their potential for use in PHA synthesis.

Production of PHA

PHAs are a class of polyesters synthesized in the form of intracellular granules by a variety of bacteria, acting as a carbon and energy reserve (Anjum et al. 2016). PHA-producing microorganisms are divided into two groups. Group I is formed by microorganisms such as *Ralstonia eutropha* (i.e., *Alcaligenes eutrophus* or *Cupriavidus necator*) (Davis et al. 1969; Vandamme and Coenye 2004) and *Pseudomonas oleovorans* which require excess carbon and limitation of at least one of the necessary nutrients—usually phosphorus, nitrogen, oxygen or sulfur. On the other hand, group II contains microorganisms that can accumulate PHA during the growth phase, without the need for nutritional stress, such as: *A. latus*, *Azotobacter vinelandii*, and recombinant *Escherichia coli* (Lee 1996).

The type of biopolymer produced may vary depending on the carbon source and the microorganism used, as it can be produced by different metabolic pathways (Sudesh et al. 2000; Alves et al. 2017). There are three main metabolic pathways involved in the biosynthesis of PHAs. The first uses sugars as a carbon source; the second, fatty acids or the result of their degradation; and the third, the biosynthesis of fatty acids (Tsuge 2002).

For P(3HB) production to be economically viable, the microorganism must be able to accumulate at least 60% of its cell mass in polymer (Alves et al. 2017). In industrial production, the main microorganisms used to obtain P(3HB) are *C. necator* and recombinant *E. coli* (Riaz et al. 2021). *C. necator*, for example, can obtain high yields of more than 80% of its dry weight in polymer (Lee 1996).

The main production costs for this biopolymer are related to the carbon source and the extraction process. Twenty-eight to 50% of the cost refers to the carbon source (Nielsen et al. 2017) and up to 50% to the extraction (Macagnan et al. 2016). Several strategies are being studied to increase P(3HB) accumulation and develop more efficient fermentation and recovery methods (Lee 1996).

Application of SSF in the production of PHA

Three different methodologies were found in the literature to employ the solid-state fermentation (SSF) technique for the production of PHAs: solid-state fermentation (SSF)—which consists of the process in only one step in solid medium; solid-state enzymatic hydrolysis (SSEH) in which both steps take place in a solid medium (SSF + SSF), the first for the hydrolysis of the by-product and the second for the production of PHA; and solid-state fermentation (SSF) associated with submerged fermentation (SmF). In this case, one (SSF + SmF) or two steps can be performed by SSF (SSF + SSF + SmF). The hydrolysis of the by-product is carried out in a solid medium and subsequently the production of PHA is carried out in a liquid medium, combining the two techniques. In the next topics, the 3 methodologies will be detailed and compared with each other.

SSF

SSF is a bioprocess in which there is little or no free water filling the spaces between the solid particles (Thomas et al. 2013; Mitchell et al. 2002). However, it contains enough moisture to allow cellular growth and metabolism of the microorganism (Barragán et al. 2016). It may have a solid substrate that acts as a source of nutrients for the microorganisms or it may contain an inert support scaffold to which nutrients will be added (Singhania et al. 2009).

This fermentation process has various advantages including the similarity between the natural habitats of the microorganisms (Hölker And Lenz 2005) and the use of nutrients originating from agro-industrial by-products, which reduces production costs (Makkar and Cameotra 2001). This method presents lower operating costs associated with the control and monitoring of pH, agitation, temperature, and aeration (Soccol et al. 2017). Nonetheless, this fermentation process also presents some disadvantages, such as difficulties in controlling the fermentation parameters during the process (Durand and Chereau 1988; Thomas et al. 2013) and limitation in the product's recovery and purification steps (Srivastava et al. 2019).

According to Singhania et al. (2009), the use of SSF was limited in scale-up studies. However, biochemical engineering has increased the number of studies focused on the design of bioreactors. Arora et al. (2018) and Manan and Webb (2017) discuss the design of bioreactors specifically for SSF.

Although there is a large number of studies regarding the use of SSF in the production of different bioproducts, only a few reports its application in PHA production (Sirohi et al. 2020). Castillo et al. (2009) compared the use of by-products in PHA production between solid and submerged fermentation processes. They found that further studies on the application of SSF were needed, especially in the development of bioreactors and more efficient fermentation strategies.

Sindhu et al. (2015) reviewed the topic and found that SSF has advantages over SmF for the production of PHA. However, improvements in the optimization of processes and parameters are still needed for their industrial implementation. Koller (2018), Li and Wilkins (2020) and Sirohi et al. (2020) also cited the SSF processes while reviewing the advances in the substrates and by-products used in the

production of PHA. According to Sirohi et al. (2020), SSF remains poorly described for the production of P(3HB).

One of the first studies to address SSF in the production of PHA was by Oliveira et al. (2004). They used agro-industrial by-products such as soy cake and babassu cake as support, or added glucose and sugar cane molasses. The maximum production reached was 4.9 mg_{PHB}/g_{medium}. Oliveira et al. (2007) verified that the P(3HB) produced from the methodology of Oliveira et al. (2004) showed thermal properties similar to those produced by SmF. Therefore, highlighting the SSF process as a viable alternative for P(3HB) production.

The first study reported that used Polyurethane Foam (PUF) as an inert support substance in SSF for PHA production, specifically for PHB, was published by Ramadas et al. (2013). PUF is porous and capable of absorbing water. PUF was added with a hydrolyzate containing nutrients, trace elements solution and *Bacillus sphaericus* NII. The use of PUF facilitates the removal of cell biomass, an important feature in the production of P(3HB) since it is intracellular. The authors reported that after optimizing the process through response surface analysis, maximum PHB yield was observed. Using an inoculum of 8×10^8 CFU/mL, 1.7% (w/v), $(NH_4)_2SO_4$ and pH 9.5, 0.169 g/g of P(3HB) was produced. The authors also found that the increase in cellular biomass was not associated with the increase in P(3HB) synthesis but related to a higher ratio of carbon to nitrogen in the culture medium.

Sathiyanarayanan et al. (2013) showed that the industrial by-product of tapioca, palm jaggery, horse gram flour and trace element solution showed a PHB production of 55% before optimization. The results showed that the trace element solution and the gram flour were limiting factors, increasing the PHB production. They had a maximum result of 8.7 g/kg (56.8%) of PHB. The authors characterized the biopolymer produced with potential for use in biomedical applications.

Naranje et al. (2016) studied the use of agricultural by-products as a substrate for the cultivation of *B. megaterium*. The maximum PHB were obtained using wheat bran and cotton seed oil cake. The highest production was found after adding ammonium chloride (NH_4Cl) to wheat bran (2.72 mg/mL). Therefore, NH_4Cl was presented as a promising source of nitrogen in PHB production by *B. megaterium*.

Sharma and Bajaj (2016) evaluated the potential of *B. cereus* PS10 in SSF. The authors used malt enriched with mineral salt solution (MSS) as a substrate, obtaining 14.4 mg/g of PHB in 48 h. According to these authors, the SSF isolate, and process demonstrate potential for the synthesis of P(3HB). Pati et al. (2020) studied SSF in mineral salt medium (MSM) with agar–agar (2%) and obtained maximum values of 1.56 g/L of PHB. These results were higher when compared to SmF (0.60 g/L). Additionally, P(3HB) in this study had high cytocompatibility, fast biodegradation in soil, and potential biochemical applications. As well as Pati et al. (2020), Mohapatra et al. (2020) also evaluated modified minimal salt agar medium (MSM) comparing SSF and SmF. This study showed maximum values of 3.72 g/L by SSF and 2.31 g/L by SmF.

As shown on Table 1 the main focus of the studies published between 2004 and 2016 was the use of agro-industrial by-products as substrate and support, whether they were added for nutrient supplementation. The study by Ramadas et al. (2013) was the only one to analyze the use of an inert support substance, Polyurethane Foam (PUF), in addition to agro-industrial by-products, concluding that SSF presents itself as a fermentative process with potential in P(3HB) production. Furthermore, it is similar to the already commercialized P(3HB) that is produced submerged, with high molecular weight and low crystallinity (Oliveira et al. 2007). Its range of applications is increased by these characteristics, like their biomedical applicability (Sathiyanarayanan et al. 2013; Pati et al. 2020; Mohapatra et al. 2020).

SSF variations in PHA production

The SSF process, composed of a single step, presents as an alternative to SmF in PHA production. However, it is still necessary to optimize the process so that it can be used industrially. In this context, the studies search for methodologies that use SSF variations. In these methodologies, hydrolysis of agro-industrial by-products are carried out by SSF. This hydrolyzate is then used as an industrial substrate to produce PHB by SSF or SmF. Figure 1 shows the different fermented methodologies presented in literature.

Hydrolysis by SSF associated with production by SmF

Some researchers (Table 2) describe the use of a methodology composed of two stages, associating SSF and SmF fermentations (Fig. 1e). The 1st stage uses SSF for the hydrolysis of biomass and formation of an extract rich in crude enzymes. The 2nd step uses the hydrolyzate formed in the 1st as a substrate for PHA synthesis through SmF.

Koutinas et al. (2013) used *A. awamori* for the formation of an enzyme-rich hydrolyzate. Jerusalem artichoke (JA) tubers was used as substrate for PHB synthesis by SmF. The final product was 4 g/L with an initial concentration of free amino nitrogen (FAN) of 0.43 mg/L. The increase in the concentration of FAN from dry cell mass and the increase in PHB demonstrate that high levels of FAN facilitate microbial growth. Another characteristic was the accumulation of PHB that occurred during cell growth. The authors attributed this result to the oxygen limitation that can occur when fermentation is carried out in Erlenmeyer flasks.

Similarly, Kachrimanidou et al. (2013) used sunflower meal as a substrate to produce crude enzymes using *A.*

Table 1 Scientific studies that used solid-state fermentation (SSF) for PHA production

Microorganism	Culture medium (support/carbon source e/or substrate)[a]	Fermentation scale	Time to PHA_{max} (h)[a]	Type of PHA	PHA $percentage_{max}$ (%)[a]	PHA $yield_{máx}$ (g/L ou g/kg)[a]	Productivity[b]	References
Ralstonia eutropha DSM 545	Soy cake/sugar cane molasses 2.5% (w/w)	Erlenmeyer	60 h	P(3HB)	39%	4.9 g/kg	0.082 g/kg/h[c]	Oliveira et al. (2004)
Ralstonia eutropha DSM 545	Soy cake/sugar cane molasses 2.5% (w/w)	Erlenmeyer	36 h	P(3HB)	33.3%	3.1 g/kg	0.086 g/kg/h[d]	Oliveira et al. (2007)
Bacillus sphaericus NII 0838	Polyurethane Foam (PUF)/ jackfruit seed powder hydrolyzate (JS)	Erlenmeyer	96 h	P(3HB)	–	0.169 g/g	0.0017 g/g/h[d]	Ramadas et al. (2013)
Bacillus megaterium MSBN04	Palm jaggery/ horse gram flour and trace element solution	Erlenmeyer	–	PHB	56.81%	8.637 g/kg	0.180 g/kg/h[e]	Sathiyanarayanan et al. (2013)
Bacillus megaterium	Wheat bran/ solid state culture media (SSC)	Erlenmeyer	24 h	PHB	–	2.72 g/L	0.113 g/L/h[d]	Naranje et al. (2016)
Bacillus cereus PS 10	Malt/mineral salt solution (MSS)	Erlenmeyer	48 h	P(3HB)	–	14.4 g/kg	0.300 g/kg/h[e]	Sharma and Bajaj (2016)
Bacillus megaterium OUAT 016	Ágar-agar/ Modified minimal salt medium (MSM)	Petri dish	72 h	PHB-co-PHV	62%	3.72 g/L	0.052 g/L/h[d]	Mohapatra et al. (2020)
Bacillus sp. C1	Ágar-agar (2%)/mineral salt medium (MSM)	Petri dish	72 h	PHB	35.53%	1.56 g/L	0.022 g/L/h[e]	Pati et al. (2020)

[a]The culture medium, time, PHA production and yield data were filled from the best results found by the authors

[b]According to Castilho et al. (2009) productivity is defined as the concentration of PHB produced, divided by the time required to produce this concentration

[c]Productivity by Castilho et al. (2009)

[d]Calculated in this review from the proposed by Castilho et al. (2009)

[e]Martínez-Avila et al. (2021) calculated the productivity based on the work of Castilho et al. (2009)

oryzae and *C. necator* DSM 545 for production by SmF. As a result, 9.9 g/L^{-1} of P(3HB-co-3HV) was obtained. The production was concomitant with cell growth and FAN consumption (0.54 g/L). Similarly, to Koutinas et al. (2013), they considered oxygen a limiting factor. Additionally, the increase in the sunflower meal hydrolyzate used in SSF interfered in the amount of crude enzymes produced, such as proteolytic ones. Consequently, increasing the amount of FAN present in the SmF.

Another study was carried out by García et al. (2013) in which the SSF step used *A. oryzae* and two biodiesel by-products: crude glycerol and rapeseed meal as a carbon source. The hydrolyzate was used as a substrate for the synthesis of P(3HB-co-3HV) by SmF. The maximum production was 10.9 g/L. The results showed that FAN concentrations of 0.5 g/L favored microbial growth, but reduced P(3HB-co-3HV) accumulation. Biopolymer production, microbial growth, and consumption of FAN occurred simultaneously, ceasing its synthesis with total consumption of FAN. The authors attributed this fact to decreasing oxygen present in Erlenmeyer flasks as a possible inducer of PHA synthesis, as did Koutinas et al. (2013) and Kachrimanidou et al. (2013).

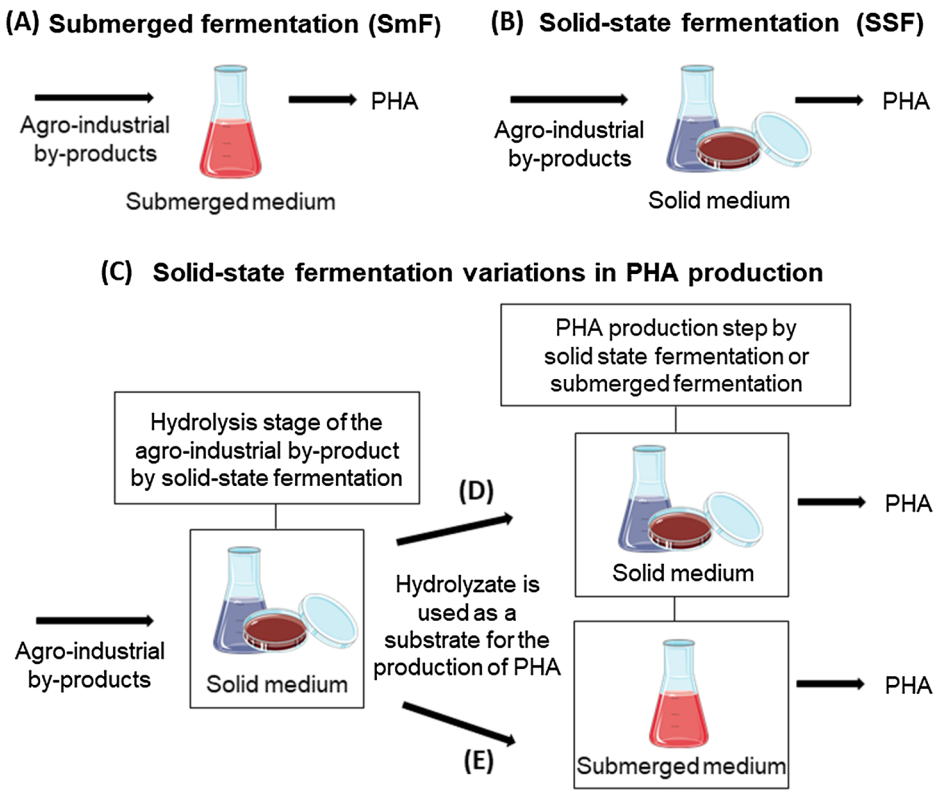

Fig. 1 Difference between fermentation methodologies for PHA production. **A** Submerged fermentation; **B** solid-state fermentation; **C** Production of PHA in two steps: 1st step—solid-state fermentation; 2nd step—production of PHA; **D** 2nd step—solid-state fermentation; **E** 2nd step—submerged fermentation. Figure was generated using imagens from Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license (https://smart.servier.com/)

Salakkam and Webb (2018) reported the use of rapeseed meal as a nitrogen source and crude glycerol as a carbon source to produce PHB using the same methodology in the mentioned studies. SSF was performed by *A. oryzae* and PHB production by SmF. Fermentation was performed in a bioreactor, with the addition of crude or pure glycerol every 24 h. Production of 25 g/L and 24.7 g/L of PHB were obtained, respectively.

Kachrimanidou et al. (2014) optimized the fractionation process of fractions rich in proteins, antioxidants and enzymes as supplements for use as a culture medium. The method was similar to the previously mentioned studies, using *A. oryzae* to form the sunflower meal hydrolyzate, and after that, *C. necator* DSM 7237 for PHB synthesis by SmF. Twenty-seven g/L of PHB were produced in a bioreactor with a glycerol feed and a FAN concentration of 0.58 g/L. The authors found that the concentration of FAN and inorganic phosphorus (IP) interfered with the accumulation of PHB. Therefore, inducing PHB accumulation with IP as a limiting factor. Although *C. necator* DSM 7237 only produces P(3HB), the authors found that levulinic acid (LA) acted as a precursor to produce P(3HB-co-3HV).

Dimou et al. (2015) evaluated wine lees (WL) and their fractionation as a substrate for PHB production. WL also produced antioxidants, tartrate and ethanol. The highest PHB production with and without the addition of minerals were 10.7 g/L and 30.1 g/L, respectively, with a FAN concentration of 0.7 g/L. The accumulation of PHB occurred after the total consumption of IP, which acted as a limiting factor, as indicated in the study by Kachrimanidou et al. (2014). In addition, they observed that the production is significantly influenced by FAN concentration. High FAN concentration (0.5 g/L) in Erlenmeyers decreases the amount of PHB. In the bioreactor, the concentration with the highest production was 0.7 g/L, decreasing when the concentration reached 0.95 g/L.

Kachrimanidou et al. (2015) used the same conditions and evaluated the use of three concentrations of sunflower meal hydrolysates (I, II and III), which can interfere with the final production of PHB (Kachrimanidou et al. 2013). Hydrolyzate I have initial FAN concentrations of 0.41 g/L. In the first 6 h of submerged fermentation, PHB production occurred simultaneously with cell growth and FAN and IP consumption. After total IP consumption, both PHB production and cell growth ceased, resulting in 1.2 g/L of PHB. Hydrolyzate II has higher concentrations of FAN of 0.704 g/L, producing 66.7% of PHB after 54 h. Hydrolyzate III has initial FAN concentrations of 0.74 g/L. The accumulation of PHB started after the total consumption of IP. Phosphorus was a limiting factor, with PHB reaching 57 g/L (86.2%), which is higher than the other two hydrolysates.

Kachrimanidou et al. (2016) used sunflower meal hydrolyzate added with crude glycerol and LA as precursor of P(3HB-co-3HV) and initial FAN concentrations of 0.6 g/L.

Table 2 Studies using the SSF Hydrolysis methodology associated with SmF for the production of PHA

Microorganism	Culture medium (support and/or carbon source)[a]	Fermentation scale/substrate (g or mL)	PHA production mode	Time to PHAmax (h)[a]	Type of PHA	PHA percentage$_{máx}$ (%)[a]	PHA yield$_{máx}$[a]	Productivity (g/L/h)[b]	References
Cupriavidus necator DSM 4058	Jerusalem artichoke tubers (JA) yeast extract	Erlenmeyer/5 g of substrate	–	56 h	PHB	51.9%	4 g/L	0.071[c]	Koutinas et al. (2013)
Cupriavidus necator DSM 545	Rapeseed meal + crude glicerol	Erlenmeyer/50 mL	Fed-Batch	–	P(3HB-co-3HV)	55.6%	10.9 g/L	0.12[d]	García et al. (2013)
Cupriavidus necator DSM 545	Sunflower meal + crude glicerol	Erlenmeyer/50 mL	Fed-Batch		P(3HB-co-3HV)	50%	9.9 g/L	0.09[d]	Kachrimanidou et al. (2013)
Cupriavidus necator DSM4058	Rapeseed meal + crude glicerol	Bioreactor/1000 mL	Fed-Batch	120 h	PHB	82.3%	24.7 g/L	0.21	Salakkam and Webb (2018)
Cupriavidus necator DSM 7237	Sunflower meal + crude glicerol	Bioreactor/800 mL	Fed-Batch	98 h	PHB	72.9%	27 g/L	0.28	Kachrimanidou et al. (2014)
Cupriavidus necator DSM 7237	Sunflower meal + crude glicerol + levulinic acid	Bioreactor/800 mL	Fed-Batch	53 h	P(3HB-co-3HV)	66.4%	23.4 g/L	0.24	Kachrimanidou et al. (2014)
Cupriavidus necator DSM 7237	Wine lees (WL) + crude glicerol	Bioreactor/–	Fed-Batch	54 h	PHB	71.3%	30.1 g/L	0.56	Dimou et al. (2015)
Cupriavidus necator DSM 7237	Sunflower meal + crude glicerol	Bioreactor/800 mL	Fed-Batch	–	PHB	86.2%	57 g/L	0.4	Kachrimanidou et al. (2015)
Cupriavidus necator DSM 7237	Sunflower meal + crude glicerol + levulinic acid	Bioreactor/800 mL	–	–	P(3HB-co-3HV)	78.9%	–	–	Kachrimanidou et al. (2016)

[a]The culture medium, time, PHA production and yield data were filled from the best results found by the authors

[b]According to Castilho et al. (2009) productivity is defined as the concentration of PHB produced, divided by the time required to produce this concentration

[c]Calculated in this review from the proposed by Castilho et al. (2009)

[d]Productivity by Kachrimanidou et al. (2014)

The final maximum P(3HB-co-3HV) production was 78.9%. This occurred due to the exhaustion of phosphorus in the medium. Lysed cells were used as a nutrient for a new production of PHB. Glycerol was added as a carbon source to provide sustainability in a biorefinery concept capable of reducing the cost of PHA production and recovery.

When using Erlenmeyer flasks, optimal FAN concentrations approximated 0.4 g/L and the accumulation of PHA was concomitant with microbial growth (Koutinas et al. 2013; García et al. 2013; Kachrimanidou et al. 2013). In a bioreactor, the highest yields were obtained at approximately 0.7 g/L FAN (Dimou et al. 2015; Kachrimanidou et al. 2014, 2015—hydrolyzate III). Lower or higher values, such as 0.41 g/L (Kachrimanidou et al. 2015—hydrolyzate I) and 0.95 g/L (Dimou et al. 2015) can decrease production. In addition, IP acted as a limiting factor and inducer of polymer synthesis.

These results suggest that the initial SSF step responsible for the hydrolysis of by-products can interfere with the final amount of nutrients present in the hydrolyzate, in the Carbon/FAN ratio. Consequently, this can interfere with the final production of PHA by SmF. Therefore, this methodology should be further optimized to reduce costs in the PHA production process.

Solid-state fermentation containing two steps in solid medium

Martínez-Avila et al. (2021) described the use of a process, classified by the authors as solid-state enzymatic hydrolysis

(SSEH) (Fig. 1d). In this process, two steps occur in solid medium. First, a microorganism carries out the SSF, for the hydrolysis of the substrate. Then, the second step is carried out by a PHA-producing microorganism. This step is also composed of SSF. The enzymatic hydrolysis of the substrates was carried out using brewer's spent grain (BSG), grape pomace (GP) and olive-mill solid waste (OSW), from enzymatic extract produced by *Aspergillus niger*. Subsequently, the hydrolysates were used as substrates to produce PHA by *C. necator* or *Burkholderia cepacia*. The authors compared the production between the SSEH (Fig. 1d) and SSF (Fig. 1b) methodologies.

The results showed that *B. cepacia* presented higher production results than *C. necator*. In addition, SSEH promoted an increase in PHA yields. For BSG, GP and OSW, yields increased 54%, 41% and 31%, respectively. The maximum yield found was from the BSG hydrolyzate with 12.5 mg/g^{-1}, composed of P(3HB) (92%), (PH2MV) (7%) and (P3HV) (1%). Comparing the two methodologies, the authors found that applying two stages of SSEH yielded a higher percentage of production than a single stage SSF process.

Recently, Llimós et al. (2022) reported a new study evaluating a different methodology, associating 3 steps: two for SSF and one for SmF. The 1st step of SSF is responsible for producing an extract rich in enzymes; in the 2nd step by SSF, the enzymes produced in step 1 are used for hydrolysis of the substrate. And the 3rd stage is the production of PHA, by SmF. Therefore, associating the two techniques previously described, 1—associating two stages of SSF and 2—associating SSF and SmF, as shown in Fig. 2.

PHA production was performed using the microorganisms *B. cepacia* and *C. necator*. The substrate used for the 2nd stage by SSF was brewer's spent grain (BSG). In the end, the authors verified that *B. cepacia* had higher sugar consumption but lower PHA production (7.0 ± 0.6 mg/g) than *C. necator* (9.0 ± 0.6 mg/g). Comparing the two studies, in the work of Martínez-Avila et al. (2021), *C. necator* had a lower production of PHA, in relation to *B. cepacia* which presented the highest PHA production (12.5 mg/g^{-1}). On the other hand, Llimós et al. (2022) found that *C. necator* had higher PHA production than *B. cepacia*, as shown in Table 3.

The main difference between the two methodologies is about the medium of the production of PHA. Martínez-Avila et al. (2021) used a solid medium [SSF + SSF] while in the study by Llimós et al. (2022) PHA production was a liquid

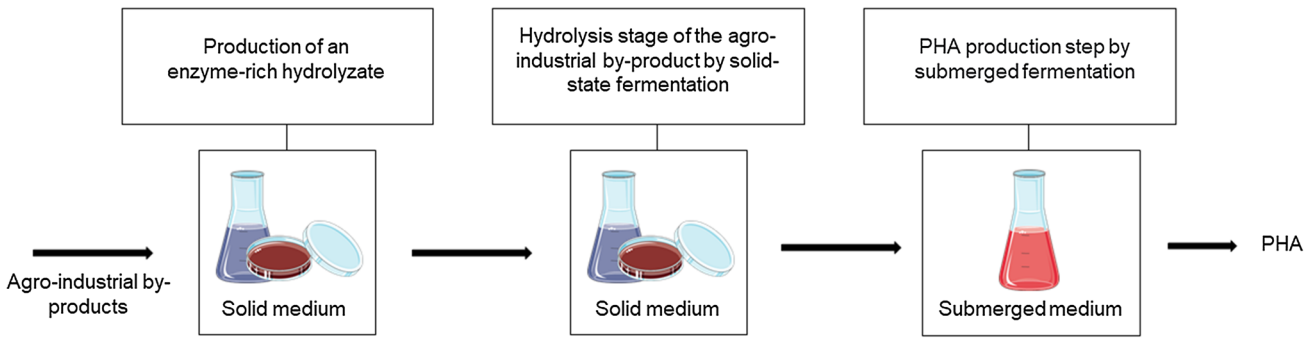

Fig. 2 Methodology associating SSF + SSF + SmF proposed by Llimós et al. (2022). Figure was generated using imagens from Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license (https://smart.servier.com/)

Table 3 Comparison between studies that associate two steps by SSF + SSF

Microorganism	Methodology	PHA percentage$_{máx}$ (%)	PHA yield$_{máx}$	Productivity (g/kg/h)[a]	References
Cupriavidus necator (DSM428)	SSF + SSF	41	–	–	Martínez-Avila et al. (2021)
Burkholderia cepacia (CCM 2656)	SSF + SSF	36.2	12.5 mg/g^{-1}	0.33	Martínez-Avila et al. (2021)
Burkholderia cepacia (CCM 2656)	SSF + SSF + SmF	–	7.0 ± 0.6 mg/g	0.097[b]	Llimós et al. (2022)
Cupriavidus necator (DSM428)	SSF + SSF + SmF	–	9.0 ± 0.44 mg/g	0.187[b]	Llimós et al. (2022)

[a]According to Castilho et al. (2009) productivity is defined as the concentration of PHB produced, divided by the time required to produce this concentration

[b]Calculated in this review from the proposed by Castilho et al. (2009)

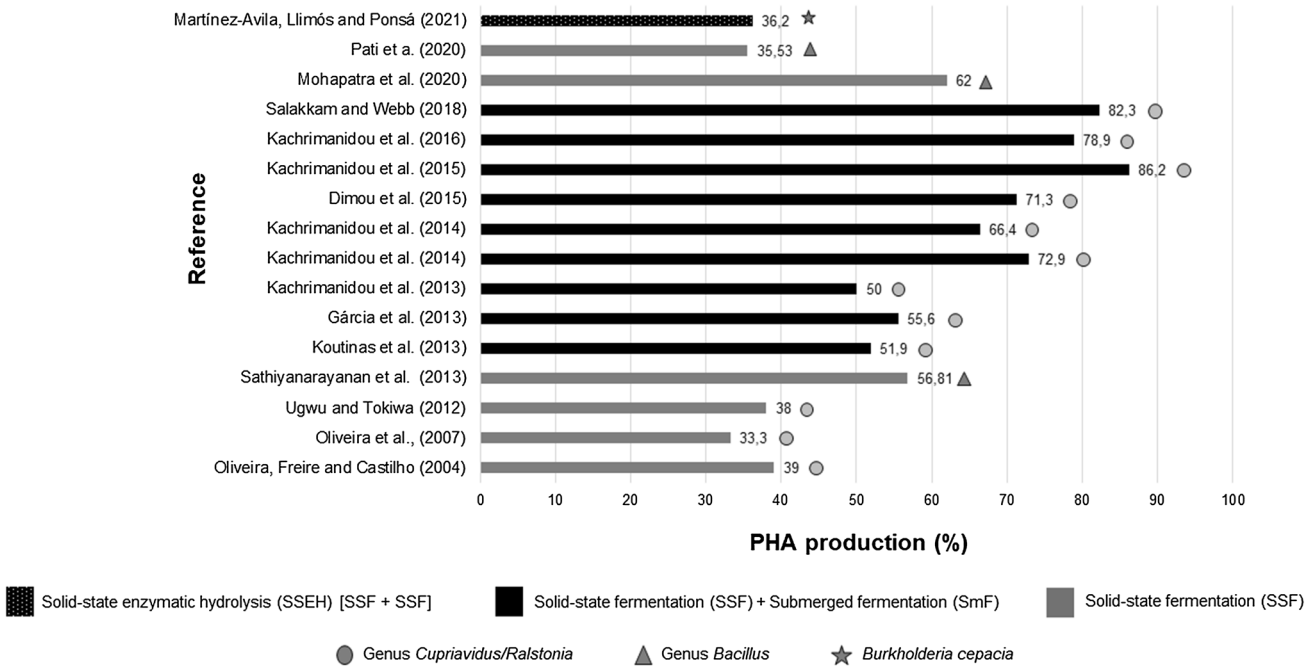

Fig. 3 Maximum PHA (%) production for the different methodologies that expressed the results in percentage. Black bars represent studies that use two production stages: first: SSF and second: SmF. Black bar with white dots represents SSEH. Gray bars represent SSF with only one step in solid culture. Gray circles identify studies that used microorganisms in the *Cupriavidus/Ralstonia* genus. Gray triangles identify studies that used microorganisms in the *Bacillus* genus. The gray star identifies the study that used the *Burkholderia cepacia* microorganism

medium [SSF + SSF + SmF]. The fact that *C. necator* presented lower yield production in solid medium (Martínez-Avila et al. 2021) and higher in the submerged medium compared with *B. cepacia*, may demonstrate that the solid medium may not be the best form of growth for this microorganism. Martínez-Avila et al. (2021) describe that this result suggests that *B. cepacia* was better than *C. necator* to exploit the available nutrients in solid medium for cell growth and PHA production.

Comparison between different methodologies of SSF application in PHA production

Regarding the production percentage data, the highest results of the traditional SSF methodology were 62% (Mohapatra et al. 2020) and 56.8% (Sathiyanarayanan et al. 2013), as shown in Fig. 3. On the other hand, variations of SSF methodologies that combine SSF and SmF had the highest PHA percentage of 86.2% (Kachrimanidou et al. 2015) and 82.3% (Salakkam and Webb 2018) (Fig. 3). The SSEH methodology that uses the two steps (SSF + SSF) obtained 36.2% (Martínez-Avila et al. 2021).

The comparative analysis was carried out based on results of production percentage of PHA (%) as it is the most used unit among the studies. The SSEH and SSF studies used mass units (g) while the SSF with SmF studies used volume (mL/L) to calculate production results. This hinders accurate comparisons between processes highlighting the need to standardize the ways in which results are reported. In addition, the highest percentage results do not necessarily represent highest production and productivity. Alves et al. (2017) found that some studies obtained high percentage concentration, that is, high intracellular accumulation, and low P(3HB) yield. According to the authors this could be due to a low accumulation of P(3HB), unfavorable operating parameters or an inefficient extraction method.

The studies that associated SSF and SmF used *C. necator*. Microorganisms of the *Bacillus* genus and *C. necator* were evaluated in the traditional SSF methodology, while *C. necator* and *Burkholderia cepacia* were used in the SSEH (SSF + SSF) method. Among all the methodologies evaluated, the highest percentage results were found by associating SSF and SmF, using *C. necator*. Although *C. necator* is considered the main producer of P(3HB) (Bhatia et al. 2018), in the traditional SSF process, the highest percentage results were found in studies that used microorganisms of the *Bacillus* genus.

This difference suggests that the type of fermentation process may interfere with cellular metabolism and with

characteristics of the bioproduct (Oliveira et al. 2007). Other factors such as temperature, pH, substrates and microorganisms can interfere with SSF. One of the main factors that contributes to this is the activity of available water (AW), which can modify the microorganism's metabolic production and process of excretion (Sindhu et al. 2015).

Access to nutrients may have been a limiting factor in traditional SSF without hydrolysis, since wild type *C. necator* cannot use sucrose as a carbon source for PHB synthesis (Park et al. 2014; Bhatia et al. 2018). This bacterium cannot use glucose, lactose and galactose (Raberg et al. 2018), with fructose being the main carbon source (Bhatia et al. 2018; Reinecke and Steinbüchel 2008). However, sucrose can be hydrolyzed into fructose and glucose by the enzyme invertase (Yang and Montgomery 2007).

In the study by Martínez-Avila et al. (2021), the hydrolysis of the by-product and the production of PHA were performed by SSF, using SSEH [SSF + SSF]. Nevertheless, *C. necator* produced lower yields than the other microorganism evaluated (*B. cepacia*). As opposed to Martínez-Avila et al. (2021) study, the methodologies associating SSF and SmF synthesize PHA in a submerged medium and hydrolyze the by-product. This suggests that, in addition to the hydrolysis process, the production strategy is a crucial factor that can interfere with the microorganism's production. These results suggest that *C. necator* is not the most suitable microorganism for PHA production through SSF.

All reviewed studies that associated solid and submerged cultivation, carried out the hydrolysis process of agro-industrial by-products. This may have facilitated the metabolism by *C. necator*. For example, the hydrolyzate from JA tubers was mostly composed of polysaccharide inulin. This inulin was hydrolyzed into fructose and sucrose by inulinase, and the sucrose was converted into glucose by the enzyme invertase. Additionally, it contains free reducing sugars, proteins, and minerals (Koutinas et al. 2013).

The sunflower meal hydrolyzate is obtained from the extraction of sunflower seed oils. It is rich in proteins, and its composition may vary according to the cultivation conditions and industrial process employed (Kachrimanidou et al. 2013). *C. necator* can use vegetable oils, which consist of triacylglycerols, as carbon sources, (Brigham et al. 2010) to produce acetyl-coa intermediates (Brigham et al. 2012). Another example is crude glycerol which has free fatty acids in its composition (Kachrimanidou et al. 2014; García et al. 2013). These can be used through the β-oxidation pathway to generate PHA precursors (Riedel et al. 2013).

However, in SSF, the *Bacillus* genus presented the highest percentage results. Naranje, Wadhe and Muddeshwar (2016) concluded that *B. megaterium* showed potential for further investigation on PHB production with solid-state medium. Literature reports that some microorganisms of the *Bacillus* genus can use a wide variety of carbon sources (Tsuge et al. 2015), including raw agricultural materials (Halami 2008). In the case of *B. megaterium*, carbon sources include sugarcane molasses, maltose; xylose, sodium gluconate, glucose (Gouda et al. 2001), sucrose (Faccin et al. 2009; Mohanrasu et al. 2020), Whey (Israni et al. 2020), and hydrolysates of agricultural by-products, such as corn straw, sugarcane bagasse, banana stem (Dañez et al. 2020), glycerol, sodium acetate, mannitol and starch (Mohanrasu et al. 2020).

Furthermore, the equipment used can interfere with bioproduct synthesis and microbial metabolism. Some authors mention the possibility of reducing the amount of oxygen present in Erlenmeyer flasks to act as an inducer of PHA synthesis by *C. necator* simultaneously with cell growth (Koutinas et al. 2013; García et al. 2013; Kachrimanidou et al. 2013). Faccin et al. (2009) reported that P(3HB) production by *B. megaterium* in a bioreactor showed a reduction of 30% compared to Erlenmeyer flasks under the same conditions. Thus, suggesting that using the bioreactor has favored cell growth over P(3HB) synthesis and that an increase in oxygen availability may decrease P(3HB) synthesis in this species.

Alternatively, this may be due to the difference in the biosynthesis process between both microorganisms. As mentioned, *C. necator* is classified in group I, requiring limitation of nutrients to produce PHA (Alves et al. 2017). According to Lee (1996), group I microorganisms generally present better production using a two-step batch system. This allows large cellular production leading to nutrient limitation and allowing the production of PHA. Nonetheless, premature nutrient limitation can result in low cell quantity, therefore, low PHA production (Lee 1996).

Although literature does not mention in which group *Bacillus* is classified, *B. megaterium* showed PHB production associated with cell growth (Dañez et al. 2020; McCool et al. 1996; Omar et al. 2001). Furthermore, nitrogen limitation was not required for production (Faccin et al. 2009). The studies by Thakur et al. (2001) and Borah et al. (2002) reported similar characteristics using *B. mycoides*. All these characteristics belong to microorganisms of group II. According to Lee (1996), group II shows better results when submitted to continuous feeding systems containing nitrogen-rich supplements, to increase cell production and PHA synthesis (Lee 1996). However, in these studies, the highest production percentages found by SSF using *Bacillus* were achieved using a discontinuous process.

Bacillus sp. is suitable in studies that use SSF for PHA synthesis, while *C. necator* was more suitable in studies using SmF. In addition, associating the SSF and SmF processes and adding a by-product hydrolysis step showed potential for studies in PHA production with *C. necator*. As for SSEH (SSF + SSF), further research is required with

microorganisms in the *Bacillus* genus to compare the results of the other methodologies mentioned in this review.

The use of by-products and enzymes is suggested to increase PHA production by *C. necator* in the SSF fermentation process. However, their use can increase the cost of the process. A microbial consortium is an alternative capable of hydrolyzing the carbohydrate used and generating free sugars (Bhatia et al. 2018). This alternative is used in studies that associate SSF with SmF, which employ the hydrolysis of by-products by fungi of the *Aspergillus* genus through SSF. However, these do not produce PHA. On the other hand, microorganisms of the *Bacillus* genus can hydrolyze by-products and still synthesize PHA. This highlights the possibility of using *Bacillus* sp. and *C. necator* combined in the SSF process.

Bhatia et al. (2018) evaluated a consortium composed of *Bacillus subtilis* and *Ralstonia eutropha* 5119 in submerged fermentation. Other studies also evaluate the use of microbial consortiums, co-cultures, mixed cultures or mixed microbial cultures (MMC) in PHA production (Sindhu et al. 2020; Subramanian et al. 2019; Löwe et al. 2017; Shalin et al. 2014). Additionally, several reviews discuss the use of mixed cultures in PHA production (Reis et al. 2003; Dias et al. 2006; Serafim et al. 2008). Recent review papers by Pakalapati et al. (2018) and Li and Wilkins (2020) cite the use of SSF as a recent and expanding process in PHA production. However, they do not cite any studies linking it to the use of mixed cultures.

Although most works describe percentage of PHA, as mentioned above, it does not necessarily represent a higher production. According to Cabrera et al. (2019), productivity is the parameter that has the greatest criterion to evaluate the operational conditions of a process, whereas a high productivity for biomass is a relevant parameter for scaling of the process. Liu et al. (2021) describes that to produce PHA an industrial scale at a lower cost, high productivity and yield of PHA from a cheap carbon source is required. Thus, taking into account the industrial process, the best unit to be used should be the PHA productivity, in addition to being a measure that involves process time and production costs, which can be a more comparable data between solid (SSF)

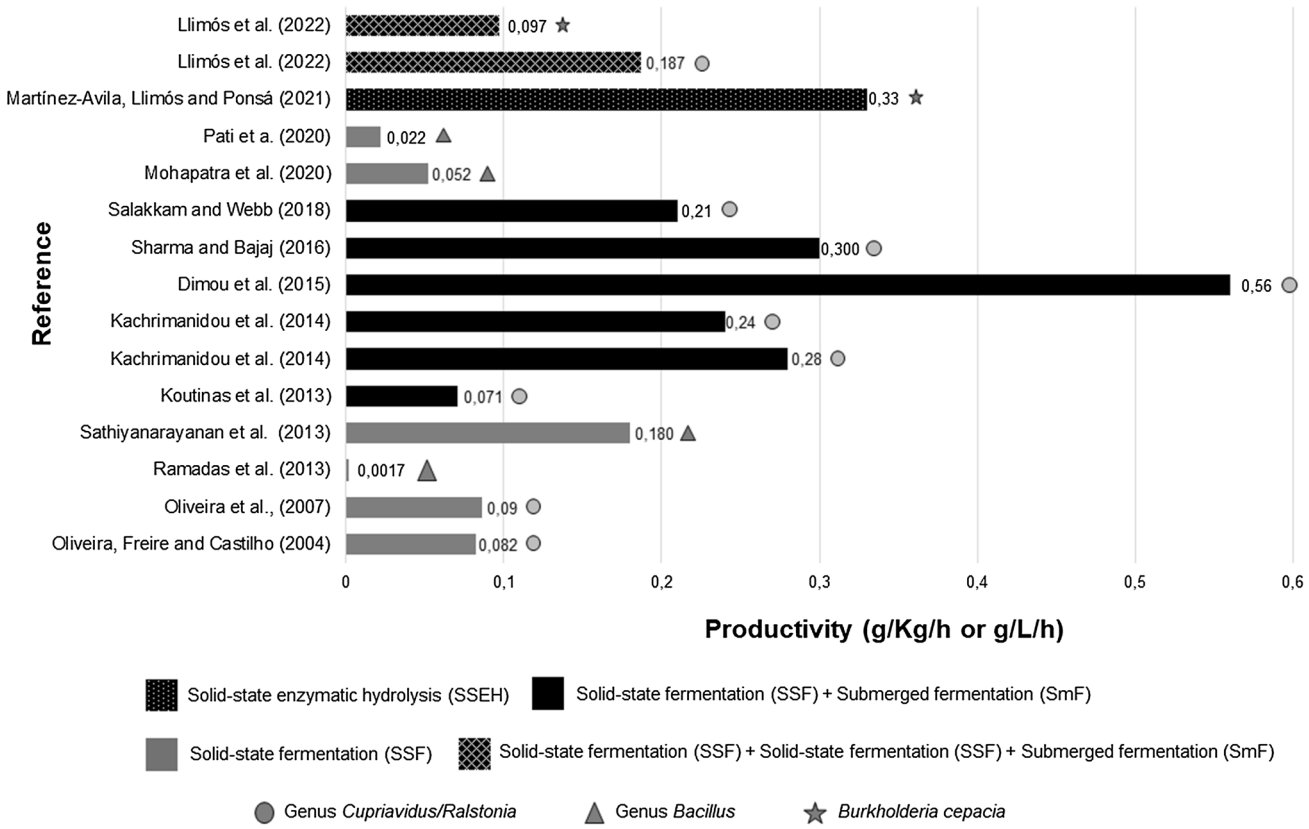

Fig. 4 Productivity results presented in this review and calculated as proposed by Castilho et al. (2009). Black bars represent studies that use two production stages: first: SSF and second: SmF. Black bar with white dots represents SSEH. Black bar with white lines represents the 3-stage methodology (SSF + SSF + SmF). Gray bars represent SSF with only one step in solid culture. Gray circles identify studies that used microorganisms in the *Cupriavidus/Ralstonia* genus. Gray triangles identify studies that used microorganisms in the *Bacillus* genus. The gray star identifies the study that used the *Burkholderia cepacia* microorganism. Figure made in Microsoft Excel® 2019

and liquid (SmF) methodologies, since it is a data that refers to the entire process.

However, not all studies report this data. Therefore, Castilho et al. (2009) proposed to calculate the productivity as: "the concentration of PHB produced (in this case PHA), divided by the time required to produce this concentration". This definition was used as a basis by Martínez-Avila et al. (2021) and used to calculate and compare productivity as well as PHA (%) results among studies in this review (Fig. 4).

Comparing the productivity results with the PHA (%), it can be seen that *C. necator* remains with the highest production, using the methodology that associates SSF and SmF. However, the studies with highest productivity are not necessarily the ones with the highest percentage. The studies by Kachrimanidou et al. (2014) and Salakam and Webb (2018) were among the highest PHA (%), 72.9% and 82.3%, respectively. But when analyzing productivity, the highest production was in the work of Dimou et al. (2015) (0.56 g/L/h), which had a lower PHA (%), 71.3%.

In SSF, the highest results of PHA (%) were using the genus *Bacillus*. Mohapatra et al. (2020) with 62% and Sathiyanarayanan et al. (2013) with 56.81%. However, looking at the productivity data, only Sathiyanarayanan et al. (2013) showed higher productivity (0.180 g/L/h) than studies that used *C. necator* (Oliveira et al. 2007 and Oliveira et al. 2004). This demonstrates that the percentage data alone do not represent the amount of PHA produced in a process.

Finally, Llimós et al. (2022) proposes a 3-step process (SSF + SSF + SmF). However, with these results, it is not possible to state whether the addition of one more step of SSF for the hydrolysis of the substrate separately from the crude enzyme production process increased the PHA production enough to justify its use, taking into account the cost of the process. This is because *B. cepacia* showed higher productivity in two steps methodology (SSF + SSF), and lower in three (SSF + SSF + SmF). On the other hand, it is difficult to use *C. necator* for this comparison, since the solid medium is not ideal for its growth. Other microorganisms could be evaluated, such as those of the genus *Bacillus*, which showed potential for growth and production of PHA by SSF.

What does the literature say about innovation and industrial application of SSF for PHA production?

López-Gómez and Venus (2021) discuss the combine use of SmF and SSF techniques for the production of bioproducts, aiming to reduce their cost. Both techniques combine the advantage of using agro-industrial by-products by SSF and the downstream process by SmF. Although it is not the scope of the present study, and to the best of our knowledge, this was the only review found in the literature, that discusses and cites the association of SSF and SmF techniques for the production of PHA.

About industrial application, Blunt et al. (2018) describes that most of the works that seek to improve the productivity of the PHA production process by SmF are carried out on a laboratory scale with up to 5 L, with few studies described in the literature that analyze the scaling up process. Recently, studies have been published in the literature evaluating the production of PHA on a pilot scale by SmF (Gutschmann et al. 2022; Schmid et al. 2021; Morgan-Sagastume et al. 2020).

Concerning SSF, Martínez-Avila et al. (2021) was the first work to evaluate the SSF scaling up process for the production of PHA, obtaining a production of 9.5 mg/g and productivity of 0.132 g/kg/h. The authors report that although bench-scale production was lower when compared to laboratory-scale production, it is still competitive with other systems. The SSF scaling process is still a problem that makes it difficult to implement this system at an industrial level (Martínez-Avila et al. 2022). The fact that this work is the first described in the literature demonstrates the need for further studies on the SSF scaling process for the production of PHA.

Thus, it can be seen that the association between the two methodologies (SSF + SmF) is an innovative process that has been gaining ground in recent years. Despite this, there is a lack of studies that evaluate the use of SSF for the production of PHA at the bench and at pilot level, as well as technological studies that seek its commercial and industrial application for PHA production. There is also a need for studies to develop new methodologies, evaluate a greater range of microorganisms, and influence of fermentation parameters.

Conclusion

Solid-state fermentation (SSF) has advantages, such as the use of agro-industrial by-products, which can reduce the costs of substrates used in PHA production. Nonetheless, there are still few studies that report PHA synthesis in solid culture medium compared to SmF. Despite the advantages, SSF still presents challenges, such as homogenization, analysis of substrate consumption, nutrients and cell growth. Moreover, cell metabolism during the process requires more understanding in order to optimize the design, operation, and scaling of bioreactors.

This review describes three different methodologies that apply the SSF process in the production of PHA. Two are for PHA production in solid culture medium: SSF and SSEH. The

third production method uses SmF associated with an SSF stage to increase PHA production. The association between the two processes presented the highest percentage and productivity results. Therefore, this methodology could be an effective alternative to increase the productivity of the process. The evaluated results suggest that *C. necator* is most suitable for processes where PHA production is carried out in SmF. Furthermore, the *Bacillus* genus has a promising potential in SSF. This is likely related to the carbon source, metabolism, and the type of fermentation process. The combine use of *C. necator* and the *Bacillus* genus in PHA production by SSF can be a promising strategy to increase PHA production.

It is important to discuss the need to standardize the way results are reported in literature to enable the comparison of different methodologies with greater accuracy, and comparing these with the SmF process. Comparing the results, it can be seen that the highest percentage results are not necessarily the ones with the highest productivity. The volumetric productivity is suggested in this review as the best parameter to be used as a comparison between the different methodologies, and for the analysis of the scaling up potential of the process. Finally, it also discusses the few studies on the combine use of these two methodologies, and the scaling-up of the PHA production process by SSF.

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Data availability Data sharing not applicable to this article as no datasets were generated during the current study. All data analyzed in the study are from the literature and are available in the article in addition to being cited in the references section.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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