



High Cell Density Culture of Dairy *Propionibacterium* sp. and *Acidipropionibacterium* sp.: A Review for Food Industry Applications

Dener Acosta de Assis¹ · Camille Machado¹ · Carla Matte¹ · Marco Antônio Záchia Ayub¹

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Abstract

The dairy bacteria *Propionibacterium* sp. and *Acidipropionibacterium* sp. are versatile and potentially probiotic microorganisms showing outstanding functionalities for the food industry, such as the production of propionic acid and vitamin B₁₂ biosynthesis. They are the only food grade microorganisms able to produce vitamin B₁₂. However, the fermentation batch process using these bacteria present some bioprocess limitations due to strong end-product inhibition, cells slow-growing rates, low product titer, yields and productivities, which reduces the bioprocess prospects for industrial applications. The high cell density culture (HCDC) bioprocess system is known as an efficient approach to overcome most of those problems. The main techniques applied to achieve HCDC of dairy *Propionibacterium* are the fed-batch cultivation, cell recycling, perfusion, extractive fermentation, and immobilization. In this review, the techniques available and reported to achieve HCDC of *Propionibacterium* sp. and *Acidipropionibacterium* sp. are discussed, and the advantages and drawbacks of this system of cultivation in relation to biomass formation, vitamin B₁₂ biosynthesis, and propionic acid production are evaluated.

Keywords Dairy propionic acid bacteria · Bioprocess technology · High cell density culture · Vitamin B₁₂ · Propionic acid · Probiotics

Introduction

Propionibacterium sp. is a rod-shaped, gram-positive, facultative anaerobe bacterium, traditionally divided based on its habitat into *classic* dairy-related species and *cutaneous*, skin-related species (Thierry et al., 2011). The single *Propionibacterium* genus was recently taxonomic reclassified into four genera: *Propionibacterium*, *Acidipropionibacterium*, *Cutibacterium*, and *Pseudopropionibacterium* (Scholz & Kilian, 2016). Dairy *Propionibacterium* sp. and *Acidipropionibacterium* sp. (dairy PAB) comprise the most relevant genera to the food industry due to their role in Swiss cheese ripening, vitamin B₁₂ biosynthesis, propionic acid production, as well as their potential probiotic properties when

added to foods (Chamlagain, 2016; Rabah et al., 2017; Wang et al., 2014; Yang et al., 2018).

Dairy PAB are the only food grade microorganisms presenting the Generally Recognized As Safe (GRAS) standard in the USA, the Qualified Presumption of Safety (QPS) status in the EU, the National Food Safety Standards of the NHS in China, and the Ministerial Ordinance on Milk and Milk products Concerning Compositional Standards in Japan able to produce vitamin B₁₂ (Fang et al., 2017). Vitamin B₁₂ is essential for human energetic metabolism and DNA replication (Nielsen et al., 2012). Its daily recommended intake is 2.4 µg, and it is important to avoid neurological and physiological disorders, specially anemia (Allen, 2008; Green et al., 2017). Usually, active vitamin B₁₂ is found in appreciable amounts only in foods of animal origin (Bitto et al., 2018; Okamoto et al., 2021; Watanabe et al., 2013). However, it is now clear that to mitigate climate change, humans will have to drastically reduce the consumption of animal-origin foods in the near future (Poore & Nemecek, 2018). Thus, it will be a rise in the demand for alternative vitamin B₁₂ sources, which can be obtained by in situ fortification of plant-based

✉ Marco Antônio Záchia Ayub
mazayub@ufrgs.br

¹ Biotechnology & Biochemical Engineering Laboratory (BiotecLab), Food Science and Technology Institute, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9500, PO Box 15090, ZC 91501-970 Porto Alegre, RS, Brazil

foods with dairy PAB (Chamlagain et al., 2018; Xie et al., 2021).

PAB present the natural ability to produce propionic acid through the Wood-Werkman cycle, which is an important property for its bio-based production (Wang & Yang, 2013; Yang et al., 2018). Propionic acid and its calcium, potassium, and ammonium salts find several applications in the food industry, as preservative and flavoring agents (Himmi et al., 2000). Additionally, it is a valuable chemical for pharmaceutical, cosmetic, and agricultural applications. Currently, this organic acid is mainly produced via petrochemical synthesis ($> 4 \times 10^5$ tons per year) due to cost considerations (Jiang et al., 2015; Piwowarek et al., 2019). However, the growing market for bio-based products, the increase in petroleum prices, and environmental issues turned its biosynthesis into a desirable option, specially under the biorefinery concept, using renewable feedstocks or industrial wastes such as molasses, residual glycerol from biodiesel, among others, for its bioproduction (Saini et al., 2019; Yang et al., 2018).

Recently, dairy PAB were also being researched for probiotic applications. Probiotics are live microorganisms that, when administered in adequate amounts, provide health benefits to the host (Hill et al., 2014). Dairy PAB have promising probiotic characteristics such as like immunomodulatory and anti-inflammatory activities, short-chain fatty acids production (SCFA), microbiota modulation, and resistance to gastrointestinal conditions (Amadoro et al., 2018; Kouya et al., 2007; Rabah et al., 2018). Their immunomodulatory and anti-inflammatory activities can attenuate non-communicable diseases (NCDs) like intestinal bowel diseases (IBDs) (Plé et al., 2015; Uchida & Mogami, 2005). These health benefits could potentially prevent the worst effects of SARS-CoV-2 infection as well (Antunes et al., 2020; Singh & Rao, 2021), further stressing the importance to keep a healthy gut microbiota, showing the need to further developments of new functional fermented products or probiotic supplements containing these next-generation therapeutic bacteria (Douillard & Vos, 2019).

Dairy PAB are versatile microorganisms presenting few nutritional requirements, capable to metabolize several carbon sources such as glucose, xylose, molasses, and residual glycerol (Coral et al., 2008; Dishisha et al., 2013; Wang et al., 2014; Yang et al., 2018). However, the growth of these bacteria under batch process systems has some performance limitations due to strong end-product inhibition, slow-growing cells, low product titer, yields and productivities, which reduces the bioprocess outcomes, thus limiting their application at industrial scale (Ahmadi et al., 2017a; Coral et al., 2008; Liang et al., 2012; Ozadali et al., 1996).

On the other hand, high cell density culture (HCDC) is an efficient approach to overcome most of the problems related to batch system, highly improving the bioprocess

efficiency (Westman & Franzén, 2015; Yang et al., 2018). In this review, the bioprocess techniques available to achieve high cell density cultures of dairy *Propionibacterium* sp. and *Acidipropionibacterium* sp. are presented, and their advantages and drawbacks in relation to biomass formation, vitamin B₁₂ synthesis, and propionic acid production are evaluated.

Bioprocess Parameters

Reviewed in this section are the key bioprocess parameters that influence the HCDC of *Propionibacterium* sp., with the impacts in the production of vitamin B₁₂ and propionic acid, namely, strain selection, growth media, temperature, pH, and aeration conditions. Additionally, a topic on mathematical modeling and statistical optimization for the bioprocess improvement is also presented.

Selection of Microorganisms

Microorganisms can be isolated from natural sources, purchased from certified collections, or obtained by bio-engineering approaches (random mutagenesis, CRISPR-Cas-mediate genome edition, among others) (Campaniello et al., 2015; Douillard & Vos, 2019). In general, the characteristics required for a competitive bioprocess are high efficiency of substrate conversion into desirable products, low susceptibility towards by-product formation (especially those causing growth arrest), microbial physiologic stability, minimal nutritional requirements, growth in low-cost media culture, and desirable production of extracellular products (Hedayati et al., 2020; Schmidell et al., 2001; Wang & Yang, 2013).

The *Propionibacterium* and *Acidipropionibacterium* genera are comprised of the following species: *P. freudenreichii*, *P. australiense*, *P. cyclohexanicum*, *P. acidifaciens*, *A. acidipropionici*, *A. jensenii*, *A. thoenii*, *A. microaerophilum*, *A. damnosum*, and *A. olivae* (Scholz & Kilian, 2016). Each one of these bacterium present different functionalities in relation to vitamin B₁₂ synthesis and propionic acid production, as well as probiotic characteristics. For instance, *P. freudenreichii* and *A. acidipropionici* have the natural capacity to produce high amounts of vitamin B₁₂ (0.2 to 1 mg g⁻¹ biomass) and propionic acid (> 50 g L⁻¹), respectively (Martens et al., 2002; Miyano et al., 2000; Wang & Yang, 2013; Yang et al., 2018).

In relation to probiotic applications, attributes such as survival towards gastrointestinal environment, absence of genes of virulence, susceptibility towards antibiotics, adhesion to epithelial cells, and immunomodulation and other health effects are strain-dependent within PAB. These characteristics were extensively reviewed elsewhere (Rabah et al., 2017). Thus, PAB strains must be screened

and certified before any probiotic claim (Hill et al., 2014). Amadoro et al. (2018) reported that *P. freudenreichii* S-1-P stimulated anti-inflammatory response of human peripheral blood mononuclear cells (PBMC). Plé et al. (2015) demonstrated that immunomodulation and anti-inflammatory activity of *P. freudenreichii* CIRM BIA 129 attenuate TNBS induced colitis in animal models. Additionally, *P. freudenreichii* CIRM BIA 129 cultured in a hyperosmotic environment ($> 1500 \text{ mosmol kg}^{-1}$) showed great viability keeping around 70% of viable cells after bile salts stress, being a promising probiotic strain (Huang et al., 2016). Omics techniques, such as proteomic and genomic, can provide helpful data for screening and selection of new functional PAB strains (Douillard & Vos, 2019). Overall, *P. freudenreichii* and *A. acidipropionici* are the most relevant species to the food industry; thus, they will be mentioned as dairy propionic acid bacteria (dairy PAB).

Growth Media Composition

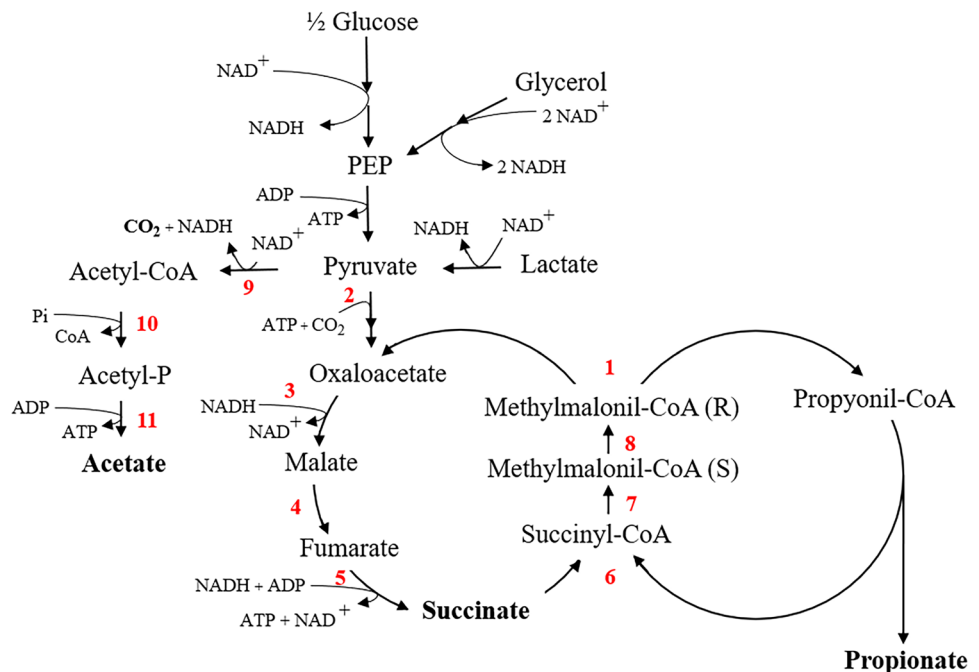
Dairy PAB growth media are usually composed by a carbon source (20–50 g L⁻¹), nitrogen source (5–15 g L⁻¹), few micronutrients (e.g., Mg²⁺, Mn²⁺, PO₄³⁻) (1–1000 mg L⁻¹), and purged with nitrogen before the inoculation process to provide anaerobiosis (Ahmadi et al., 2017b; Goswami & Srivastava, 2000; Liu et al., 2011; Martínez-Campos & de la Torre, 2002). In addition, cobalt ions and 5,6-dimethylbenzimidazole (DMBI), which are vitamin B₁₂ precursors, are of fundamental importance for active vitamin B₁₂ biosynthesis (Assis et al., 2020; Deptula et al., 2015; Hugenschmidt et al., 2011; Vandamme & Revuelta,

2016). For in situ fortification of food products, the addition of DMBI is undesirable. Thus, in these cases, reduced flavin mononucleotide (FMNH₂), a food-grade substrate, is added because dairy PAB can produce active B₁₂ from this compound (Chamlagain, 2016; Deptula et al., 2015).

PAB can metabolize several carbon sources, such as: sugars (sucrose, glucose, fructose, lactose, galactose, and xylose), molasses (from various sources such as sugarcane and soybeans), organic acids (lactic and glucuronic acids), fatty acids (linolenic, oleic, and palmitic), and other organic compounds such as glycerol (Coral et al., 2008; Goswami & Srivastava, 2000; Hedayati et al., 2020; Wang et al., 2014; Yang et al., 2018). Energy (ATP) and reduced co-factors are initially obtained in pyruvate production throughout glycolysis or the pentose phosphate pathway. Pyruvate is then oxidized to acetic acid and CO₂ or reduced in the Wood-Werkman Cycle (Fig. 1) into propionic acid, which are the main dairy PAB fermentation products (Thierry et al., 2011). The metabolic flow between pyruvate oxidation and reduction pathways is essential to maintain the intracellular redox balance (NAD⁺/NADH ratio) (Wang & Yang, 2013).

Glucose and complex carbon sources such as molasses and whey permeate are the best choices in order to obtain high dairy PAB cell density (Boyaval & Corre, 1987; Coral et al., 2008; Feng et al., 2011; Liu et al., 2016; Ozadali et al., 1996). The carbon source also exerts influence over the propionate/acetate ratio (P/A ratio) and overall fermentation performance. For example, lactate and glucose fermentation by *P. freudenreichii* produces propionic and acetic acids usually in a P/A ratio of 2:1, while propionic acid theoretical yields from glucose is approximately 0.55 g g⁻¹ (Wang &

Fig. 1 Metabolism of *Propionibacterium* sp. using different carbon sources via the Wood-Werkman cycle. Enzymes involved in the propionate pathway: (1) methylmalonyl-CoA:oxaloacetate carboxyltransferase; (2) pyruvate carboxylase; (3) malate dehydrogenase; (4) fumarate hydratase; (5) succinate dehydrogenase; (6) propionyl-CoA:succinate CoA transferase; (7) methylmalonyl-CoA mutase; and (8) methylmalonyl-CoA epimerase. Enzymes involved in the acetate and CO₂ pathway: (9) pyruvate dehydrogenase; (10) phosphate acetyltransferase; (11) acetate kinase. PEP phosphoenolpyruvate (Piveteau, 1999; Wang & Yang, 2013; Zhang et al., 2015)



Yang, 2013). On the other hand, glycerol fermentation favors the formation of reduced compounds and propionic acid is the unique product (achieving theoretical yields of 0.80 g g^{-1}), with only traces of acetic acid (P/A ratios > 30:1) been produced (Coral et al., 2008; Himmi et al., 2000; Wang & Yang, 2013).

Cell growth rate and propionic acid productivity (0.01 and 0.20 g(L h)^{-1} , respectively) are lower using glycerol as carbon source compared to glucose or lactate, caused by cell redox imbalance (Coral et al., 2008; Wang & Yang, 2013; Wang et al., 2014; Zhang & Yang, 2009). However, these parameters can be improved by glycerol/glucose co-fermentation (Liu et al., 2011; Wang & Yang, 2013; Wang et al., 2014). Additionally, it has been reported that balanced glycerol/yeast extract ratio (3:1) could also increase biomass production ($\sim 9 \text{ g L}^{-1}$), propionic acid productivity (0.31 g(L h)^{-1}) and yields (0.63 g g^{-1}), and P/A ratio (up to 50:1) in batch process (Dishisha et al., 2015). In relation to vitamin B₁₂, it has been reported that glycerol reduces vitamin B₁₂ yields in *P. freudenreichii* cultures compared to glucose. However, glycerol co-fermentation with glucose also improves the vitamin B₁₂ yields and productivity (Wang et al., 2014).

Lactate metabolism is characteristic of these microorganisms, which is well explored in cheese ripening (Thierry et al., 2005). This carbon source stimulates the production of volatile compounds, organic acids and CO₂, desirable for ripening of Swiss-type cheeses, but the process results in less biomass accumulation since lower energy is recovered from this carbon source (Boyaval & Corre, 1987; Piveteau, 1999; Thierry et al., 2005). Dairy PAB show preference for L-lactate isomer instead of D-lactate and, in relation to propionic acid production, it can provide higher productivity compared to glycerol (Coral et al., 2008; Crow, 1986). Additionally, small pH culture variations (less than 0.5 pH units) are observed in lactate fermentation due to almost equimolar acid production, which reduces the need for using alkalis in pH-controlled bioprocess (Coral et al., 2008; Lewis & Yang, 1992).

The nitrogen source also affects the growth and productivity, thus affecting a cost-effective bioprocess development. Although dairy PAB can synthesize all amino acids needed from inorganic sources such as (NH₄)₂SO₄, organic nitrogen sources like amino acids, protein hydrolysates, yeast extract, and corn steep liquor are better growth promoters (Ahmadi et al., 2017b; Thierry et al., 2011). Corn steep liquor (CSL), a byproduct from starch processing, has been widely used in several PAB bioprocess, due to its low-cost and balanced nutritional content (amino acids, minerals, and vitamins) (Nakano et al., 1996; Quesada-Chanto et al., 1998; Wang & Yang, 2013; Yang et al., 2018). Recently, this byproduct has been proved to be an excellent nitrogen source for PAB biomass production. However, concerning the propionic acid

biosynthesis, yeast extract was considered a better option (Ahmadi et al., 2017b).

Temperature

The culture temperature influences the growth rate and metabolites production of dairy PAB, which can grow in a range of temperatures from 4.5 to 40 °C (Piwowarek et al., 2019). The growth at low temperatures is very slow, but it is desirable in the Swiss cheese production (Hofherr et al., 1983). The highest cell growth rate and products formation rates are obtained in temperatures around 30 °C (Colomban et al., 1993; Coral et al., 2008; Gorret et al., 2001).

Seshadri and Mukhopadhyay (1993) reported that specific growth rate of *A. acidipropionici* ATCC 25562 increased with temperature, from 0.05 at 26 °C up to 0.1 h⁻¹ at 34 and 37 °C. Propionic acid productivity did not increase above 30 °C, and at 37 °C, the strain increased the formation of acetic acid as byproduct reducing P/A ratio (Seshadri & Mukhopadhyay, 1993). Similarly, Farhadi et al. (2013) observed the highest propionic acid production at 30 °C in a beverage fermented by *P. freudenreichii* DSM 20270 and *L. acidophilus* LA5. Piwowarek et al. (2019) reported a small increase in propionic acid production—from 4.70 to 5.13 g L⁻¹—when increasing the growth temperature of *P. freudenreichii* T82 from 30 to 37 °C, respectively.

Recently, Hedayati et al. (2020) reported that optimum temperature for vitamin B₁₂ biosynthesis by *P. freudenreichii* PTCC1674 was 38.3 °C, under which the increase of biomass production favored vitamin B₁₂ accumulation as well (Hedayati et al., 2020). These data indicates that optimal temperature for biomass/vitamin B₁₂ production might be different from the optimal temperature for propionic acid production by PAB (Colomban et al., 1993; Coral et al., 2008; Hedayati et al., 2020; Seshadri & Mukhopadhyay, 1993).

pH

In relation to pH, dairy PAB cultures under neutral conditions, pH controlled between 6 and 7, show high cell growth rate (up to 0.12 h⁻¹). Above or below this range, the cell growth rate tends to decrease (as low as 0.06 h⁻¹ under pH 5). Also, the lag phase is extended outside neutral conditions (Marshall & Odame-Darkwah, 1995; Seshadri & Mukhopadhyay, 1993; Wang et al., 2012b; Zhuge et al., 2014). Gorret et al. (2001) reported that optimal pH range for *A. acidipropionici* DSM 4900 biomass and EPS production was within pH 6.5 to 7, based on a response surface methodology (RSM). Under acid conditions (pH 5 and 5.5), biosynthesis of propionic acid was favored compared to acetic acid, which increased P/A ratio in a batch process using *A. acidipropionici* ATCC 25562 (Seshadri & Mukhopadhyay, 1993). In uncontrolled pH

cultures, *P. freudenreichii* T82 showed drastically reduction in its growth rate and products yields when the pH fell below 5 (Piwowarek et al., 2019).

Based on these observations, recently reports have suggested that controlling the culture pH at different values (for instance, pH 6.5 for the initial 48 h and then shifting to pH 5.5 or 6) is an effective strategy to improve propionic acid yields (Zhuge et al., 2014). In the first stage, pH is set for optimum microbial growth; then, in the second stage pH is reduced to direct the cell metabolic pathway towards propionic acid formation instead of biomass and byproducts formation (Feng, Xu et al., 2010). Using this pH-shift control strategy Feng, Xu et al. (2010) were able to double propionic acid productivity to as high as $0.18 \text{ g (L h)}^{-1}$, and the P/A ratio up to 4:1 compared to cultures of *P. freudenreichii* CCTCC M207015 in which the pH was kept constant at 6.5.

Aeration

Dairy PAB are facultative anaerobe microorganisms and some strains can grow under aerobic conditions, with volumetric oxygen transference coefficient (k_{La}) as high as 61 h^{-1} and up to 50% of dissolved oxygen concentration (Quesada-Chanto et al., 1997). These microorganisms harbor a partial respiratory system with menaquinones, membrane bound enzymes, cytochromes *b* and *c* that enable them to grow in the presence of oxygen, where it is the final electron acceptor (Ye et al., 1999). Although long exposure to oxygen causes decrease in cell growth due to cytochromes and menaquinones synthesis inhibition, aerobic/microaerophilic cultures can be beneficial for biomass production due to higher ATP generation (Furuichi et al., 2006; Furuichi et al., 2006). On the other hand, propionic acid and vitamin B₁₂ production are negatively affected under aerobic conditions (Quesada-Chanto et al., 1998).

The growth under aerobic conditions changes the metabolism pattern and acetic acid becomes the main end product and, at k_{La} higher than 20 h^{-1} , propionic acid production stops (Kouya et al., 2007; Quesada-Chanto et al., 1997, 1998). The Wood-Werkman cycle (Fig. 1) is reversed in presence of oxygen and propionic acid produced under anaerobiosis is consumed under aerobic conditions producing pyruvate, which is further oxidized to acetic acid (Ye et al., 1999). Based on that, the oscillation of aeration strategy (anaerobic to aerobic and aerobic to anaerobic) along fermentation can reduce the inhibitory effect of propionic acid and enhance biomass (up to threefold) (Furuichi et al., 2006; Miyano et al., 2000; Ye et al., 1996).

In relation to vitamin B₁₂, even growth at low k_{La} (10 h^{-1} and 0% of dissolved oxygen after 1 h of fermentation), showed a reduction of near 30% of this vitamin biosynthesis compared to anaerobic growth (Quesada-Chanto et al., 1998). Dairy PAB follows the anaerobic pathway for vitamin

B₁₂ biosynthesis and aeration at the beginning of fermentation reduces the activities of key B₁₂-related enzymes (e.g., δ -aminolevulinic acid synthase and δ -aminolevulinic acid dehydratase), consequently, reducing the vitamin B₁₂ yields (Martens et al., 2002; Quesada-Chanto et al., 1997, 1998).

However, periodic fluctuations from anaerobic to aerobic and conversely, from aerobic to anaerobic along the cultivation improved vitamin B₁₂ yields (twofold) and productivity (1.4-fold) when compared to fully-anaerobic fermentation (Ye et al., 1996). Low dissolved oxygen concentrations (from 0.50 to 1 ppm) allows the propionic acid consumption and the oscillation of aeration strategy decreases the negative effect over vitamin B₁₂ biosynthesis (Miyano et al., 2000; Ye et al., 1996). Moreover, oxygen is needed to produce the structural lower ligand 5,6-dimethylbenzimidazole (DMBI) when it is not provided in the culture medium (Deptula et al., 2015).

Mathematical Modeling

Mathematical modeling is an important tool for improving the performance of complex fermentation systems (Luo et al., 2021). Modeling takes into count the effect of multiple variables and their interactions upon process outputs, making it possible to predict systems behavior in otherwise difficult experimental approaches and can be successfully applied to scaling up (El-Naggar et al., 2019). Thus, relevant modeling approaches for improving dairy PAB bioprocess were reviewed in the following subsections.

Statistical DoE

Data-driven models based on statistical design of experiments (DoE) are frequently applied for optimization of dairy PAB cultures (Assis et al., 2020; Chen et al., 2013; Hedayati et al., 2020). These experimental designs significantly reduce the number of experiments, costs, and time needed in the bioprocess development, being flexible and describing complex systems in relatively simple ways (El-Naggar et al., 2019; Luo et al., 2021). On the other hand, they do not extrapolate outcomes beyond defined levels and do not explain the mechanisms of biological phenomena (Hedayati et al., 2020; Luo et al., 2021).

Overall, Plackett and Burman design and RSM (central composite design, Box-Behnken design, among others) are chosen for screening significant variables and to predict their optimal range, respectively, aiming to maximize bioprocess yields and/or productivity (Sindhu et al., 2017). Recently, our research group reached important results using this optimization strategy where biomass and vitamin B₁₂ produced by *P. freudenreichii* ATCC 13673 were increased up to 4 and 2 folds, respectively (Assis et al., 2020).

Following the same method, Hedayati et al. (2020) found synergic interaction within DMBI, elemental solution ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) and rice bran oil (RBO) upon vitamin B_{12} biosynthesis, while DMBI against temperature interaction presented negative effects. After optimization throughout Box-Behnken design (BBD), the authors were able to increase yields of vitamin B_{12} by 14% compared to previous experiments (Hedayati et al., 2020). In another research, Chen et al. (2013) increased propionic acid production by 23% solely using a central composite design (CCD), which highlights the effectiveness of this approach in bioprocess optimization.

Mechanistic Models

Mechanistic models are another mathematical representation of bioprocess based in the first-principle mechanisms of microbial activity (e.g., cell growth and metabolism flux) and their related mathematical functions instead to exclusively experimental data (Luo et al., 2021). This approach is not as versatile as DoE, requiring extensive knowledge about the subject and substantial efforts for modeling. However, after experimental validation, it becomes a powerful tool to estimate parameters (for instance, lag time, cell growth rate, etc.), to predict microbial behavior and to develop predictive control strategies (Hashemi & Roohi, 2019; López et al., 2004; Sindhu et al., 2017; Zhu et al., 2018).

The Baranyi model successfully described the growth pattern of *P. freudenreichii* PTCC 1674 and *P. shermanii* PTCC 1661 in date syrup medium under different sonication amplitudes and exposure time (Hashemi & Roohi, 2019). In the same work, the authors proposed a Gaussian function to predict propionic acid production that showed great accuracy ($\text{adj-}R^2 > 0.99$) (Hashemi & Roohi, 2019). Goswami and Srivastava (2000) developed a mathematical model to evaluate the best substrate feeding strategy for propionic acid fermentation. The model showed that maximum substrate consumption and growth rate were reached at 35 to 40 h, then, a continuous nutrient feeding (0.04 L h^{-1}) was established at this fermentation stage increasing propionic acid productivity (0.23 to $0.4 \text{ g (L h}^{-1})$) (Goswami & Srivastava, 2000).

Interestingly, Zhu et al. (2018) enhanced vitamin B_{12} content in soy-milk using Lotka Volterra model to describe interactions between *L. reuteri* ZJ03 and *P. shermanii* ZJ01 in co-fermentation. The authors found that proper anaerobic phase (5 days), temperature ($30 \text{ }^\circ\text{C}$) and pH (7) provided the least antagonistic effects between strains, as well as enhancing vitamin B_{12} yields (up to twofold) (Zhu et al., 2018). Under those circumstances, mechanistic models are a great tool to underline the design of a successful bioprocess using dairy PAB. Further, this modeling process requires fewer experiments for parameters estimation and model validation,

which is also desirable for time and costs reduction in the development stage (Luo et al., 2021).

Bioprocess Techniques to Obtain High Cell Density Cultures of Dairy PAB

The main bioprocess techniques applied to achieve high cell density culture (HCDC) of dairy PAB are the fed-batch bioreactor (Goswami & Srivastava, 2000; Ozadali et al., 1996), cell recycling (Crespo et al., 1991; Liu et al., 2016; Miyano et al., 2000), perfusion culture (Boyaval & Corre, 1987; Hatanaka et al., 1988; Nakano et al., 1996), extractive fermentation (Jin & Yang, 1998; Wang et al., 2012b, 2014), and cell immobilization bioreactor (Feng et al., 2011; Rickert et al., 1998; Wallenius et al., 2015).

In this section, these techniques and their potential for production of biomass, propionic acid, and vitamin B_{12} by dairy *Propionibacterium* fermentations are presented. The bioprocess design of each technique is illustrated in Fig. 2. In Tables 1 and 2, are summarized their characteristics and outcomes obtained in dairy PAB cultures, respectively.

Fed-batch

Fed-batch is a bioprocess technique that consists in substrate addition into the bioreactor throughout constant, intermittent, or exponential feeding (Schmidell et al., 2001). In relation to dairy PAB cultures, common feeding strategies are as follows: constant substrate addition at a pre-established feed rate (often from 0.01 to 0.04 L h^{-1} initiated after ~ 40 h of growth) or pulses of substrate along bioprocess (Ahmadi et al., 2017a, b; Jiang et al., 2015; Ozadali et al., 1996; Zhu et al., 2010; Zhuge et al., 2014).

Ozadali et al. (1996) achieved high cell density (37 g L^{-1}) of *A. acidipropionici* P9 in a fed-batch with glucose pulses whenever its concentration was close to exhaustion in medium. The authors also reported high propionic acid yields (0.54 g g^{-1}), titer (45 g L^{-1}), and productivity ($0.31 \text{ g (L h}^{-1})$) (Ozadali et al., 1996). Similar results were obtained in fed-batch cultures using *A. acidipropionici* ATCC 4875 at constant lactose feeding (Goswami & Srivastava, 2000). On the other hand, Zhu et al. (2010) reported lower *A. acidipropionici* CGMCC 1.2230 cell density ($\sim 5 \text{ g L}^{-1}$) and propionic acid productivity ($0.20 \text{ g (L h}^{-1})$) in a fed-batch using glycerol as carbon source. However, these authors obtained high yields (0.56 g g^{-1}), titer (44.6 g L^{-1}), and P/A ratio (18:1), which is a characteristic of glycerol fermentation with this bacterium (Zhu et al., 2010).

In relation to vitamin B_{12} , it has been reported that nitrogen sources and other nutrients must be provided in the feed solution to improve biomass yields and to support an efficient HCDC in fed-batch mode operation (Liu et al., 2016; Paik &

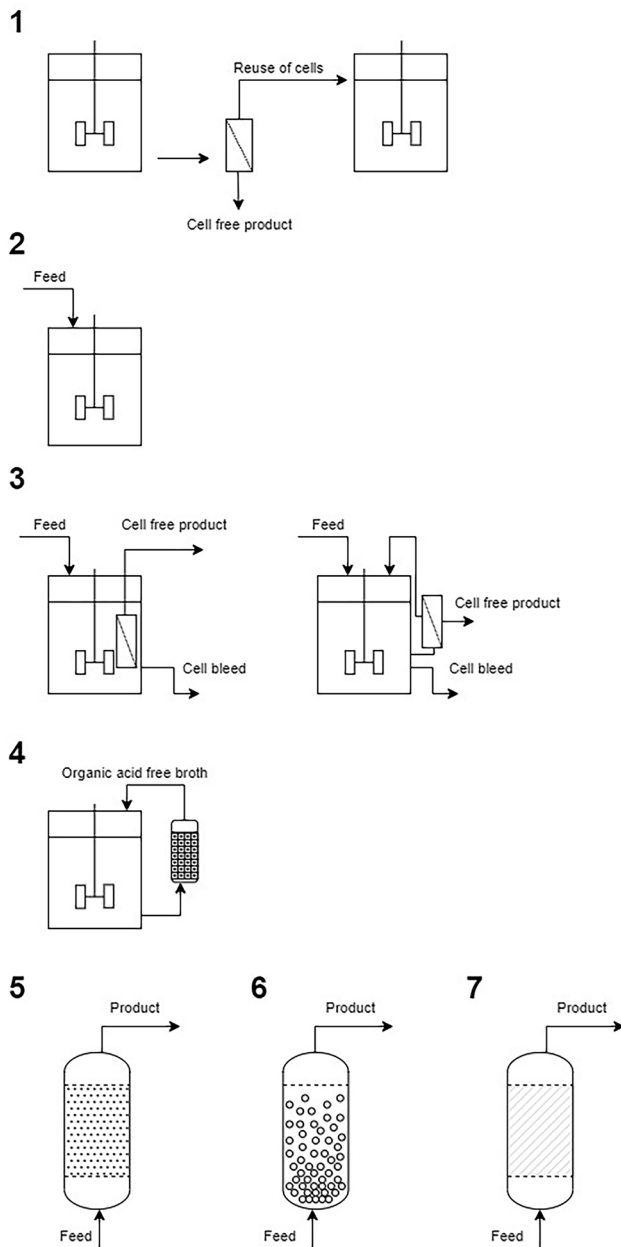


Fig. 2 Bioprocess techniques to obtain high cell density cultures of dairy PAB: (1) cell recycling; (2) fed-batch; (3) perfusion culture; (4) extractive fermentation; (5); (6); and (7) cell immobilization (fixed, expanded, and fibrous bed bioreactors, respectively)

Glatz, 1994). Biomass production is important for vitamin B₁₂ production since it is an intracellular product synthesized during the microbial growth (Martens et al., 2002). In addition, availability of vitamin B₁₂ precursors such as cobalt ions, DMBI, or FMNH₂, are also important for vitamin B₁₂ biosynthesis (Deptula et al., 2015; Hugenschmidt et al., 2011). Therefore, the composition of feeding solution should supply proper nitrogen source as well as precursors for an effective vitamin B₁₂ production in fed-batch.

Table 1 Characteristics of bioprocess techniques applied to obtain high cell density cultures of dairy PAB cultures

Bioprocess technique	Advantages	Disadvantages	References
Fed-batch	Relatively simple; avoids substrate inhibition; modulates cell growth rate and metabolic activity	End-product inhibition due to organic acids accumulation	(Feng et al., 2010; Goswami & Srivastava, 2000; Zhu et al., 2010; Zhuge et al., 2014)
Cell recycling	Reduces microbial lag phase; increases cell density; decreases end-product inhibition	Operational complexity; extra costs with membranes and mechanical stress; clogging of membranes and contamination issues	(Colomban et al., 1993; Dishisha et al., 2013, 2015; Liu et al., 2016; Miyano et al., 2000; Quesada-Chanto et al., 1994; Westman & Franzén, 2015)
Perfusion	High yields and productivities (up to 10 folds higher than batch); requirement for small equipment; and products homogeneity	Operational complexity; higher material costs (medium, membranes, harvest tanks, etc.); clogging of membranes and contamination issues	(Boyaval & Corre, 1987; Cacciuto, 2007; Crespo et al., 1991; Croughan, 2015; Pollock et al., 2013)
Extractive fermentation	Reduced end-product inhibitions; increased cell viability and reduced alkali consumption during pH control	Operational complexity; extra costs; limited extractive capacity and toxicity of resins and solvents	(Jin & Yang, 1998; Ozadali et al., 1996; Solichien et al., 1995; Wang et al., 2012a, 2014)
Immobilization	Increased cell viability (up to 10 ¹⁰ CFU g ⁻¹ support); reduced end-product inhibition and lag phase	Operational complexity; mechanical stability; diffusional limitation; contamination and difficult to scale-up	(Feng et al., 2011; Gardner & Champagne, 2005; Gu et al., 1998; Paik & Glatz, 1994; Suwannakham & Yang, 2005; Wallenius et al., 2015)

Table 2 Comparison of outcomes obtained by dairy *Propionibacterium* sp. cultured under different bioprocess techniques

Bioprocess technique	Bioprocess parameter								References
	Strain	Temperature (°C)	pH	X (g L ⁻¹)	Y _p (g g ⁻¹ substrate)	Y _{B12} (g g ⁻¹ substrate)	Q _p (g (L h ⁻¹))	Q _{B12} (mg (L h ⁻¹))	
Batch	<i>A. acidipropionici</i> ATCC 4965	30	7.0	1.8			0.07		(Boyaval & Corre, 1987)
Batch	<i>P. freudenreichii</i> IFO 12,424	30	6.7	6.9		0.98*		0.14	(Miyano et al., 2000)
Batch	<i>A. acidipropionici</i> ATCC 4875	30	6.5	14	0.44		0.25		(Goswami & Srivastava, 2001)
Batch	<i>P. freudenreichii</i> CICC 10019	30	7.0	7.9	0.44		0.20		(Wang et al., 2012b)
Batch	<i>A. acidipropionici</i> ATCC 4875	32	6.5		0.55		0.03		(Zhang & Yang, 2009)
Fed-batch	<i>A. acidipropionici</i> ATCC 4875	30	6.5	20			0.40		(Goswami & Srivastava, 2000)
Fed-batch	<i>A. acidipropionici</i> ATCC 4875	30	6.0	24	0.38		0.20		(Liu et al., 2016)
Fed-batch	<i>A. acidipropionici</i> CDBB-1049	30	7.0	10	0.48				(Martínez-Campos & de la Torre, 2002)
Fed-batch	<i>P. freudenreichii</i> CICC 10019	30	7.0		0.71	0.72	0.36	0.36	(Wang et al., 2014)
Fed-batch	<i>P. freudenreichii</i> CICC 10019	30	7.0	6	0.35	0.61	0.13	0.23	(Wang et al., 2012a)
Cell recycling	<i>A. acidipropionici</i> ATCC 4965	30	7.0	100	0.17		14.3		(Boyaval & Corre, 1987)
Cell recycling	<i>A. acidipropionici</i> DSM 8250	37	7.0	75	0.5	0.66	4.42	1	(Quesada-Chanto et al., 1994)
Cell recycling	<i>P. freudenreichii</i> IFO 12,424	30	6.7	35		0.77*		0.62	(Miyano et al., 2000)
Cell recycling	<i>A. acidipropionici</i> ATCC 25,562	37	6.0	137	0.56		10.3		(Crespo et al., 1991)
Cell recycling	<i>P. shermanii</i> PZ-3	30	6.5	227		0.23*		0.8	(Hatanaka et al., 1988)
Extractive fermentation	<i>P. freudenreichii</i> CICC 10019	30	7.0	6.8	0.78		0.45		(Wang et al., 2012b)
Extractive fermentation	<i>P. shermanii</i> PZ3	30	6.6	8.8			0.39		(Zhang et al., 1993)
Extractive fermentation	<i>P. freudenreichii</i> CICC 10019	30	7.0	6.8	0.66	0.54	0.33	0.27	(Wang et al., 2012a)
Extractive fermentation	<i>P. freudenreichii</i> CICC 10019	30	7.0		0.75	0.37	0.35	0.36	(Wang et al., 2014)
Extractive fermentation	<i>A. acidipropionici</i> ATCC 4875	30	7.1		0.66		1		(Jin & Yang, 1998)
Immobilization	<i>P. freudenreichii</i> DSM 4902	32	6.5		0.58		0.48		(Wang & Yang, 2013)
Immobilization	<i>A. acidipropionici</i> ATCC 4875 ACK-Tet	32	7.0	30–60	0.56		0.03		(Zhang & Yang, 2009)
Immobilization	<i>A. acidipropionici</i> P200910	32	7.0		0.52		0.96		(Paik & Glatz, 1994)
Immobilization	<i>A. acidipropionici</i> NRRL B-3569	32	7.0	99	0.58		0.88		(Wallenius et al., 2015)
Immobilization	<i>A. acidipropionici</i> CGMCC 1.2230	37	6.0		0.43		0.71		(Zhu et al., 2012)

Table 2 (continued)

X biomass, Y_p propionic acid yield, $Y_{B_{12}}$ vitamin B₁₂ yield, Q_p propionic acid productivity, $Q_{B_{12}}$ vitamin B₁₂ productivity

* mg g biomass⁻¹

Cell Recycling

Semi-continuous Process

In the cell recycling technique, after a batch or a fed-batch phase, microbial cells are harvested by unit operations such as centrifugation, sedimentation, or ultrafiltration, resuspended in fresh medium, and cultured for another cycle, while the previous fermented broth is forwarded to downstream steps (Colomban et al., 1993; Dishisha et al., 2015; Liu et al., 2016; Schmidell et al., 2001). Due to their characteristics (Table 1), cell recycling is considered a potential process for slow-growing microorganisms such as dairy PAB (Quesada-Chanto et al., 1994).

The highest propionic acid productivities obtained in glycerol fermentation under batch process (1 to 1.42 g (L h⁻¹)) were reported using sequential batches with cell recycling (Dishisha et al., 2013, 2015). In another research, high propionic acid titer (75 g L⁻¹) and average productivity (0.32 g (L h⁻¹)) were obtained in a fed-batch with *A. acidipropionici* ATCC 4875 using a dense inoculum (biomass ~ 14 g L⁻¹) recycled from a previous HCDC fed-batch (Liu et al., 2016). Colomban et al. (1993). This procedure kept *A. acidipropionici* ATCC 4965 cell concentration around 40 g L⁻¹ by ultrafiltration in a pilot-scale bioreactor (5 m³) and the authors reported a stable process (15 sequential batches, over 900 h of operation) with high propionic acid productivity (~ 1 g (L h⁻¹)). That biomass concentration was considered the best choice to improve the bioprocess efficiency, as well as to avoid contamination and clogging issues (Colomban et al., 1993).

One of the main advantages of cell recycling is that HCDC can be achieved from the start of the fermentation and be kept relatively constant throughout the experiment (Dishisha et al., 2013; Quesada-Chanto et al., 1994). Additionally, high propionic acid titer and productivities attainable make it a promising technique for propionic acid production in biorefineries (Dishisha et al., 2013), especially when integrated to the production of other dairy PAB products such as vitamin B₁₂ or biomass itself for probiotic or cheese starter applications (Colomban et al., 1993; Dishisha et al., 2013; Quesada-Chanto et al., 1994).

In relation to vitamin B₁₂, Miyano et al. (2000) reported a higher B₁₂ productivity (0.62 mg (L h⁻¹)) in a cell recycling system compared to batch process (0.14 mg (L h⁻¹), although lower specific yields of biomass (0.77 vs 0.98 mg g⁻¹, respectively) were obtained. Quesada-Chanto et al. (1994) developed a bioprocess based in cell

recycling for the simultaneous production of propionic acid and vitamin B₁₂ by dairy PAB. The authors kept a HCDC (biomass of approximately 75 g L⁻¹) into two connected bioreactors, one under anaerobic conditions, whereas the second was run under microaerophilic conditions, reporting high propionic acid productivity (4.42 g (L h⁻¹)) and yields (0.50 g g⁻¹), along with very high vitamin B₁₂ productivity (up to 1.50 mg (L h⁻¹)) and yields (0.66 mg g⁻¹ biomass) using sugarcane molasses in cultures of *A. acidipropionici* DSM 8250 (Quesada-Chanto et al., 1994).

Continuous Process (Perfusion)

The perfusion culture system is characterized by continuously feeding fresh medium and fermented broth harvesting whilst microbial cells are kept into the bioreactor by using different cell retention devices such as spin-filters, ultrafiltration modules, among others (Crespo et al., 1991; Nakano et al., 1993; Quesada-Chanto et al., 1994). The removal of inhibitory metabolites and cell retention enable to obtain cell density and productivities 10 times higher than in batch process (Cacciuttolo, 2007). Indeed, the best propionic acid productivities (Table 2) ever reported for PAB fermentation were obtained by using continuous process with cell retention by ultrafiltration (Boyaval & Corre, 1987; Crespo et al., 1991).

Improved results over other techniques have also been reported for biomass production in perfusion cultures (Table 2). The highest biomass obtained for dairy PAB fermentation (over 200 g L⁻¹) was reached in a perfusion process using hollow-fiber module as a cell retention device (Hatanaka et al., 1988). Additionally, this HCDC also produced high vitamin B₁₂ titer (52 mg L⁻¹) showing average specific yields of 0.23 mg g⁻¹ of biomass (Hatanaka et al., 1988).

Nakano et al. (1993), using rotative ceramic membranes as the cell retention device reported cell densities around 50 g L⁻¹ in a continuous culture of *P. freudenreichii* ATCC 8262. When the same bioprocess was coupled to a propionic acid removal system, biomass production increased to 150 g L⁻¹ (Nakano et al., 1996). The combined systems enabled the recirculation of fermented broth (propionic acid free) and residual nutrients were efficiently consumed, reducing fresh medium feeding, equivalent to 30% less glucose being supplied, compared to traditional perfusion (Nakano et al., 1996).

The pore size of membrane devices is another variable that influences yields and productivity due its role in cell

retention into the bioreactor. Goswami and Srivastava (2001) reported that the higher cell retention using 5 μm spin filter compared to 10 μm spin filter improved propionic acid yields (0.40 vs 0.35 g g^{-1}) and productivities (0.90 vs 0.53 $\text{g (L h}^{-1}\text{)}$). In addition, the rotation of devices such as spin filters and rotative ceramic membranes enhances filtration process because it reduces the cake layer formation upon membrane surface, minimizing fouling and clogging issues (Goswami & Srivastava, 2001; Nakano et al., 1993).

High cell density cultures over 100 g L^{-1} can be obtained using ultrafiltration modules, but it may turn propionic acid fermentation unstable and hinder the operation due to clogging, viscosity increase, and foam formation (Blanc & Goma, 1989; Boyaval & Corre, 1987; Crespo et al., 1991). In these cases, a cell bleed rate might be established to avoid biomass to reach critical levels and to ensure a stable continuous process (Blanc & Goma, 1989; Crespo et al., 1991). Furthermore, the harvested biomass can also be used as probiotics, cheese starter, or vitamin B₁₂ source (Colomban et al., 1993; Dishisha et al., 2013; Quesada-Chanto et al., 1994).

Currently, the development of new technologies like alternating tangential flow filtration (ATF) as cell retention device, is promising in order to overcome most of those problems and are currently being used in the pharmaceutical industry (Bielser et al., 2018; Zydney, 2021). In an ATF device, the filtration flow is periodically alternated (back and forth) by a diaphragm pump, which continuously harvest the broth while pushing cells back to the bioreactor, thus removing the microbial layer over the membrane surface (Kelly et al., 2014). Therefore, ATF filtration reduces membrane fouling, improves cell viability, medium consumption, cell density, and productivity compared to other cell retention devices previously cited (Bielser et al., 2018; Kelly et al., 2014; Zydney, 2021).

However, at present, there are no reports on perfusion cultures for dairy PAB in ATF systems. This opens the way to further research with these potential probiotic bacteria, especially for biomass and vitamin B₁₂ production.

Extractive Fermentation

Propionic acid inhibitory effects over cell cultures begin at concentration range of 5 to 10 g L^{-1} , the critical value being around 30 g L^{-1} , when cell growth is arrested (Suwannakham & Yang, 2005; Wang et al., 2012b), and propionic acid formation is also disrupted due to inhibition of key enzymes propionyl CoA transferase, and oxaloacetate carboxyltransferase (Fig. 1) (Suwannakham & Yang, 2005). This inhibition suppress the propionic acid pathway and increases byproducts formation such as acetic and succinic acids (Suwannakham & Yang, 2005).

Hence, accumulation of propionic acid along fermentation reduces its own yield and productivity (Gu et al., 1998; Suwannakham & Yang, 2005).

It has been suggested that propionic acid concentration should be kept at low level into the bioreactor ($< 10 \text{ g L}^{-1}$) in order to achieve high cell growth and products formation rate (Gu et al., 1998; Jin & Yang, 1998; Wang et al., 2012b). To do so, bioreactors can be coupled to activated charcoal-packed columns, ion-exchange columns, system of solvent-driven extraction, or electro dialysis system, which enable the selective removal of organic acids from the broth in a process called extractive fermentation or in situ product removal (ISPR) (Solichien et al., 1995; Wang et al., 2012b; Zhang et al., 1993).

High propionic acid productivity ($\sim 1 \text{ g (L h}^{-1}\text{)}$) and titer (75 g L^{-1}) were reported by Jin & Yang, (1998) using solvent driven extraction in a fed-batch fermentations of *A. acidipropionici* ATCC 4875. The authors showed that solvent toxicity could be removed if the extractor were contained in hollow-fiber membranes to reduce contact with microbial cells (Jin & Yang, 1998). Even higher propionic acid titer (91 g L^{-1}) with high yields (0.75 g g^{-1}) but lower productivity (0.36 $\text{g (L h}^{-1}\text{)}$) were reported for the fed-batch process of *P. freudenreichii* CICC 10019 using expanded bed adsorption bioreactor (EBAB) for propionic acid removal. In contrast, vitamin B₁₂ yields were reduced by approximately 50% (from 0.72 to 0.37 mg g^{-1} substrate) compared to conventional fed-batch (Wang et al., 2014). In another research, vitamin B₁₂ yields were also reduced in EBAB fed-batch compared to the batch process (0.95 to 0.37 mg g^{-1} substrate) using the same strain. However, propionic acid yields were increased (from 0.56 to 0.75 g g^{-1}) indicating a metabolic shift into propionic acid biosynthesis in these systems (Wang et al., 2020).

The literature data indicates that extractive fermentation keeping propionic acid at low concentrations ($< 10 \text{ g L}^{-1}$) is a better strategy for propionic acid production than for vitamin B₁₂ or dairy PAB biomass (Gu et al., 1998; Wang et al., 2012b, 2020; Zhang et al., 1993). However, the control of propionic acid at two levels (i.e., low at first stage and high at late stages of fermentation) along with DMBI addition strategy provided one of the highest vitamin B₁₂ titer (59.5 mg L^{-1}), yields (0.98 mg g^{-1} substrate), and productivity (0.59 $\text{mg (L h}^{-1}\text{)}$) ever reported for dairy PAB cultures (Table 2) (Wang et al., 2015). It was suggested that, at late stage of fermentation, inhibitory effects of propionic acid over its own metabolic pathway favors the nutrient shift to vitamin B₁₂ biosynthesis (Wang et al., 2015). Thus, extractive fermentation can be effective for vitamin B₁₂ production as well, but the process operation must be optimized toward vitamin B₁₂ biosynthesis instead of propionic acid formation.

Cell Immobilized Bioreactors

Immobilization technique consists in confining microbial cells into physical structures called support materials such as natural and synthetic polymers, glass beads, *lentikats*, among others, usually through support binding or entrapment methods (Schmidell et al., 2001; Zhu, 2007). After the immobilization, confined cells can be used as biocatalysts in multiple fermentation process (Dishisha et al., 2012; Feng et al., 2011; Rickert et al., 1998).

Fibrous Bed Bioreactor Immobilization

Column bioreactors, consisting of fixed, expanded, and fibrous bed bioreactors, are often chosen for fermentation using immobilized cells (Schmidell et al., 2001). In particular, fibrous bed bioreactors (FBBs) gained attention because they present less diffusional limitations and pressure issues, maintenance of high active cell density under long-term operation, reduction of downtime, system stability, and easy immobilization techniques (nonspecific adsorption and entrapment) (Feng et al., 2010; Suwannakham & Yang, 2005; Zhang & Yang, 2009; Zhu, 2007).

In this process, a microbial cell suspension is circulated throughout the column packed with spiral wound fibrous material that can be cotton, terry cloth, bagasse, among others, to enable cells to adhere onto support surface or to get entrapped into void spaces, where they will grow during the bioprocess reaching concentrations of up to 40 g L^{-1} (Yang, 1996). The major drawbacks observed in this technique are the presence of free-cells into broth ($\sim 20\%$) and additional material costs (Feng, Chen et al., 2010; Yang, 1996; Zhang & Yang, 2009).

The FBB system is self-renewing in the sense that excess of aging or dead cells are continually desorbed while new ones are allowed to grow maintaining fermentation for long periods, from months to a year, without clogging or pressure issues (Yang, 1996). Furthermore, cells immobilized into FBB have shown the capacity to modulate their membrane composition, morphological aspects and, more important, the activity of key metabolic enzymes (Suwannakham & Yang, 2005). This adaptative process strengthens propionic acid tolerance and cell viability, thus high propionic acid titer and less byproducts formation can be achieved by the adapted strains (Feng et al., 2010; Suwannakham & Yang, 2005; Zhang & Yang, 2009).

One of the highest propionic acid titers ever reported (106 g L^{-1}) was obtained in a long-term fed-batch operation ($\sim 3000 \text{ h}$) using *A. acidipropionici* 4875 ACK-Tet immobilized in FBB. However, the acid productivity was low ($< 0.04 \text{ g (L h}^{-1})$) due propionic acid accumulation (Zhang & Yang, 2009). Recently, a high trehalose producer mutant strain was able to increase propionic acid

titer (135 g L^{-1}) with increased productivity ($0.61 \text{ g (L h}^{-1})$) and yields (0.67 g g^{-1} lactose) in a fed-batch FBB system (Jiang et al., 2015). The authors suggested that higher trehalose production could enhance microbial tolerance over that propionic acid concentration (Jiang et al., 2015).

Feng et al. (2011) developed a cleaner, effective, and economical bioprocess to produce propionic acid by coupling the use of hydrolyzed cells and molasses as low-cost substrates to fed-batch fermentation with *P. freudenreichii* immobilized in plant-fibrous bed bioreactor. Despite low productivities ($0.26 \text{ g (L h}^{-1})$), they obtained high propionic acid titer ($\sim 80 \text{ g L}^{-1}$) and purity (77%), otherwise unfeasible in batch process, demonstrating the potential of the technique (Feng et al., 2011). In continuous fermentation at high dilution rates ($0.1\text{--}0.3 \text{ h}^{-1}$), better propionic acid productivities were achieved (up to $1 \text{ g (L h}^{-1})$), but generating a diluted effluent (less than 15 g L^{-1} of propionic acid) (Dishisha et al., 2012; Lewis & Yang, 1992).

Alginate Beads Immobilization

One of the most used immobilization technique is the cell entrapment in Ca-alginate supports (Schmidell et al., 2001). In this process, a microbial suspension is mixed with alginate solution (2–4% mass fraction) and then dripped into a CaCl_2 solution (2–4% mass fraction) to create a bead-shaped rigid complex that confines the microorganisms (Gardner & Champagne, 2005; Rickert et al., 1998; Xu et al., 2007). It simplifies cells recovery and reuse in repeated bioprocess, protects cells from propionic acid inhibitory effects and improves propionic acid productivity ($\sim 1 \text{ g (L h}^{-1})$ in continuous process) (Paik & Glatz, 1994). In a consecutive batch, Rickert et al. (1998) reported propionic acid productivity up to $2 \text{ g (L h}^{-1})$, attributing this good result to high initial substrate level (glucose 75 g L^{-1}) and cell density of *A. thoenii* P20 immobilized in alginate beads.

However, diffusional limitation, contamination, and, in particular, beads stability are the biggest problems to be overcome in alginate immobilization (Duarte et al., 2013). In addition, fermentations of dairy PAB immobilized in alginate beads have shown reduced vitamin B_{12} biosynthesis up to 50% compared to free cells cultures (Czaczyk et al., 1997; Gardner & Champagne, 2005; Yongsmith et al., 1982). This is attributed to the entrapment of cobalt into the alginate matrix, reducing its availability, thus affecting vitamin B_{12} biosynthesis, based on the central atom of the corrinoid ring (Czaczyk et al., 1997; Gardner & Champagne, 2005). Therefore, results gathered in the literature so far suggest that this technique seems to be more suitable for propionic acid production rather than for vitamin B_{12} .

Other Support Materials

Wallenius et al. (2015) developed an innovative xylan hydrogel matrix that supports high cell density, with estimated concentrations of 99 g L⁻¹ into the column bioreactor (~74 g of support material), providing less mass transfer problems. The authors achieved high propionic acid productivity (0.88 g (L h⁻¹)) and yields (0.58 g g⁻¹) in continuous fermentations with *A. acidipropionici* NRRL B-3569 at high dilution rate over a month of bioreactor operation. However, significant damage to the beads was observed after that period (Wallenius et al., 2015). Dishisha et al. (2012) observed that immobilization of *A. acidipropionici* DSMZ 4900 in Luffa (vegetal matrix) and Poraver beads (porous glass) was not effective, but modification of supports structures by attaching a cationic polymer such as polyethyleneimine improved the immobilization performance (Dishisha et al., 2012).

Exopolysaccharide (EPS)-producing strains have shown exceptional immobilization performance without any support modification requirements (Belgrano et al., 2018). Olguin et al. (2019) induced EPS production and biofilm formation using stress factors such as sodium chloride and citric acid to immobilize *A. acidipropionici* DSMZ 4900 in Poraver and AnoxKaldnes, a plastic support. Biofilms immobilized in Poraver material provided better propionic acid productivity (0.15–0.78 g (L h⁻¹)) in repeated batch cycles (Olguin et al., 2019). Therefore, the choice of adequate support material, immobilization technique, as well as microbial strain, growth condition and bioreactor design must be taken into count to achieve the highest fermentation performance with immobilized cells (Belgrano et al., 2018; Dishisha et al., 2012; Paik & Glatz, 1994; Rickert et al., 1998; Wallenius et al., 2015).

Conclusion

Overall, all techniques reviewed proved to be effective in improving the bioprocess performances of *Propionibacterium* sp. and *Acidipropionibacterium* sp. However, the implementation of these techniques increases, at least in some level, the bioprocess operational complexity and material costs. Therefore, the HCDC advantages and disadvantages should be taken into count before choosing a particular HCDC technique for dairy PAB fermentations. In special, fed batch using immobilized cells in fibrous bed bioreactors (FBB) appears to be a very promising technique for propionic acid production based on its relative simplicity and the possibility to produce high yields of this organic acid. On the other hand, the highest biomass production and vitamin B₁₂ biosynthesis are obtained in cell recycling systems but, unfortunately, the very promising ATF system, already in

use for some pharmaceutical applications, have not been tested for dairy PAB production, which opens the window for new research in the field. Despite its high operational complexity and costs, cell recycling could be explored to produce these outstanding microorganisms, especially for value-added applications, such as probiotic supplements and vitamin B₁₂ fortification of plant-based products. Other applications are still to be explored with the increasing interest for vitamin B₁₂ /probiotic-rich foods and beverages.

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Declarations

Conflict of Interest The authors declare no competing interests.

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