Chapter 41
Development and Scale-Up of Waste Biorefineries Systems: Lactic Acid as a Case Study

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Abstract This chapter aims to discuss relevant topics for successful development and scale-up of industrial biotechnological process in the context of a biorefinery. Lactic acid will be explored during the text since more than 90% of the world's production occurs by bioprocesses. From this perspective, some critical aspects should be considered according to the maturity level of the technology, since scaling-up investments and time estimates are not directly proportional to what is dispended at the bench level research. Misunderstandings in the design of some development stages may represent increase in investments and spent time reducing then the chances of implementing a promising technology at the industrial reality. In the following sections, a description is provided on main aspects of an industrial biotechnology and is illustrated how an integrated approach among bioprocess development, including the construction of industrial strains, fermentation, and downstream process development, along with scaling-up features and sustainability assessment, can predict possible bottlenecks and guides the research and the development of an industrial biotechnological process.

41.1 Introduction

Biological conversion of non-food and renewable feedstocks, such as lignocellulosic biomass, oil residues, industrial and municipal wastes, into a wide range of valuable bio-based products has been considered a promising approach within a waste-based biorefinery concept to promote carbon-neutral bio-economy (Ferreira et al. 2019; Ko

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et al. 2020). Bio-products or bio-based products refer to chemicals and materials derived from waste feedstocks. Examples of bio-products include enzymes, organic acids, polymers, amino acids, alcohols, fatty acids, both with wide application in the pharmaceutical, cosmetics, food, plastics, and fuels industries.

The development of a promising new technology from lab to industrial scale is a significant challenge for biotech processes as this is a high-risk venture that requires time and investment. However, it is highly recommended to have pilot plant and demo plant stages during the process development of biotech routes to validate key important technical aspects of the new process. Furthermore, process scale-up provides information for the design of the industrial equipment and generates first samples of the product, which can be after validated for a specific application. Once planned properly, scale-up can be executed successfully. This means that the new process technology can produce the target bio-product according to a required market specification in an intermediate scale matching the initial timeline and cost estimates. In other words, the success or failure of the scale-up depends on how close rate, yield, and purity at the larger scale matches up to those results from the lab bench scale. Successful scale-up also means the issues related to transport phenomena which does not appear in the lab scale are faced and resolved in a larger scale. Sometimes, even a complete understanding of the mass and heat transport phenomena was not achieved, a background for future development has been established (Reisman 1993).

The scale-up of industrial process often takes place in two stages if there is a high degree of novelty in the process. The first stage is the pilot plant with fermenters and the required downstream equipment. Frequently, at this phase, the process is not yet fully integrated, and each unit operation is operated batch-wise. The second stage is the demonstration plant with higher fermenters and the respective downstream. At this phase, the process runs continuously and has the same equipment that will be used at the industrial plant. The unit operations run fully integrated considering all the recycling streams, and the feedstock is the same that will be used in commercial unit. Sometimes, if there is low degree of novelty, demo plant may be skipped (Crater and Lievense 2018).

The time required to complete the transition from lab bench scale to manufacturing process is typically 3–10 years, which means the biotech developments are long-time projects with high financial risk. Scaling may require from 6 months to 3 years, depending on if a facility exists or need to be constructed and on the degree of novelty introduced by the new process. Any deterioration in process performance during the scale-up will be costly and can lead to the project failure. Even a short deviation, such as 4–10% of underperformance or delays (6–12 months), can significantly reduce financial results of the endeavor (Crater and Lievense 2018). This reinforces the need to have a good approach with a good planning, and an experienced team before going to the scale-up. Some important points for a good scale-up, such as think about full-scale from the start and perform the preliminary techno-economic analysis, will be stated here. Many experts in biotech scale-up state that it is important to "begin with the end in mind," which means the final industrial

full large scale should be thought of before experimental work begins. Start from the large-scale operation and not from lab scale work (Noorman and Heijnen 2017).

A realistic view of how the process looks like at production scales provides key inputs to guide the research program. Beginning with the end in mind allows the teams to prepare a detailed conceptual design of the envisioned manufacturing process. Even working with many unknown, taking realistic premises, it is possible to build process flow diagrams, material and energy balances, unit operation designs, and techno-economic models. With this approach, some problems can be anticipated, increasing the chances for success, expenses are reduced, and scale-up can be faster (Crater and Lievense 2018).

The techno-economic analysis (TEA) at the early stage of the industrial project gives the first feeling of the economic attractiveness to develop a bio-based product. TEA is also important to understand the critical performance metrics such as fermentation titer, productivity, yield, and downstream recovery that affects the rentability of the project. Every time there is a significative new input from the research, the economic model must be updated. TEA drives the go/no-go decisions for next steps of the scale-up and prioritizes goals and efforts for the research.

Usually, the development of upstream, midstream and downstream processes is carried out in separate groups. However, these teams must interact. From the beginning of the research, it is important to develop the entire process from the raw material to the final bio-product. All processes are intricately connected to each other. For example, the microorganism selected for the bioprocess significantly impacts the conduction of the development of the fermentation process, as well the yield and titer of the bio-product that can be obtained. The culture medium used in the fermentation can affect the efficiency and cost of the downstream process. Through collaborative problem solving, whole team can find solutions to overcome the barriers during the development program.

A successful case of a biotechnological process application is the bio-based lactic acid production. Lactic acid is a molecule widely used in cosmetic, chemical, food, textile, leather, pharmaceutical, and polymer industries to produce PLA (polylactic acid), which is a biodegradable and renewable polymer. It can be obtained by two routes, chemical synthesis or fermentation. The chemical synthesis results in racemic mixture of DL-lactic acid, while the fermentation route results in an optically pure L-lactic acid. The latter is metabolized by humans and finds applications in food and pharmaceutical industries and can be used in the synthesis of the biodegradable PLA polymer (Babele and Young 2020; Komesu et al. 2017a; Oliveira et al. 2018). Considering the current production of lactic acid, over 90% of the commercial production is performed via fermentation due to several advantages over the chemical synthesis route, such as the use of low-cost and renewable feedstocks, lower energy consumption, and the likelihood of obtaining high optical purity of the acid, depending on the strains used (Djukić-Vuković et al. 2012; Singhvi et al. 2018).

The following sections in this chapter will discuss relevant aspects of the development and scaling-up industrial biotechnological process, considering four synergic steps: (1) upstream process that develops robust strains capable of producing a desired bio-products using waste as renewable resources; (2) midstream process that

optimizes the bio-product synthesis through fermentations/cultivations; (3) downstream process that recovers and purifies the desired bio-product to achieve industrial level requirements; (4) sustainability assessment that guides the whole development to have a good trade-off between economic and environmental aspects. Lactic acid will be used as background to discuss these steps and highlight the importance of this integrative development.

41.2 Bioprocess Upstream—Industrial Strains Development

Microorganisms isolated from nature can produce a variety of interesting bio-products (e.g., antibiotics, enzymes, amino acids, lipids, polymers, organic acids, fuels), and for this reason, they have been employed by industry for over 100 years. However, these native strains hardly meet industrial demands because of some undesirable characteristics, including (1) unable to produce high titers and yields of the target bio-product, (2) not optimized to consume a wide variety of carbon sources, (3) low tolerance to toxic components present in bio-based feedstocks and industrially relevant stress, (4) can produce high by-products titers, which make the downstream process complicated and expensive (Yu et al. 2020; Zhang et al. 2011).

To overcome these general challenges and fully exploit the potential of microorganisms, intense efforts have been made by academia and industry to synergistically develop high-performance strains and customized bioprocesses capable of efficiently converting waste-derived carbon sources into bio-products in industrial level.

Industrial strain development requires several strategies and decision points, as discussed in excellent reviews and perspectives (Ko et al. 2020; Lee and Kim 2015). The early stage of this development includes the selection of a suitable host microorganism (also known as chassis, microbial cell factory or platform microbial). This choice is not trivial and depends on the type of product, on several physiological factors, such as capability of utilizing carbon sources, abundance of key intracellular precursors, growth in an inexpensive medium, oxygen requirement (anaerobic versus aerobic microorganisms), in addition to techno-economic, regional legal, and regulatory factors intrinsic to an industrial process (Lee and Kim 2015; Liu et al. 2020).

In recent years, the rapid progress of tools and strategies for strain optimization has opened new avenues for the development of high-performance strains, enabling the production of high "TRY" (Titer, Rate, and Yield), high level of optical purity, low nutritional supply, the use of complex agro-industrial wastes, or minimal by-products production during industrial fermentation and downstream steps. Here, we show relevant classical and advanced metabolic engineering approaches that have been evaluated to develop an increasing number of industrial strains using lactic acid production as a successful case study.

As already mentioned, the selection of a suitable host is also crucial to meet the commercial requirements of lactic acid production. Although a wide range of microorganisms can naturally produce lactic acid, the most commonly used by the industry is the homofermentative lactic acid bacteria, due to their ability to convert over 95% of sugar into optical pure lactic acid, with a maximum theoretical yield of 2 mol of acid per mol of glucose and 1.67 of acid per mol of xylose (Singhvi et al. 2018; Wang et al. 2015). Among the homofermentative bacteria, the genus *Lactobacillus* and *Bacillus* are the predominant chassis for commercial production. Some of the advantages of these strains are: (1) can naturally produce optically pure lactic acid as the primary metabolic end-product with high yield and productivity; (2) consume hexoses, disaccharides and pentoses, enabling the use of several different renewable substrates, such whey, starch, molasse; lignocellulosic materials; (3) most of them are Generally Recognized As Safe (GRAS status) (Hofvendahl and Hahn-Hägerdal 2000; Klotz et al. 2016). However, one of the major problems associated with bio-based production of lactic acid by lactic acid bacteria has been its reduced tolerance to low pH levels. To avoid the pH drop by lactic acid production and prevent growth inhibition, neutralizing agents are frequently employed to maintain a neutral pH during fermentation. However, at pH around 5–7, a substantial proportion of the product exists in lactate form (since pKa ~3.8 at 25 °C). During lactic acid purification, acidification step is required to recover the free lactic acid, increasing the cost of the process and generating a large amount of waste, such as gypsum that poses economic and environmental problems (Datta and Henry 2006; Lee and Kim 2015; Singhvi et al. 2018). In addition, lactic acid bacteria generally require complex nutrients due to their inefficiency in naturally synthesizing B vitamins and amino acids, essential components for their growth and lactic acid production (Komesu et al. 2017a; Wang et al. 2015). In general, according to Tejayadi and Cheryan (1995), the culture media, especially nitrogen supplementation, can represent about 38% of the capital costs of a bioprocess (CAPEX).

To overcome these current limitations in industrial bio-based lactic acid production and several other bio-products, traditional non-GMO (non-Genetically Modified Organism) strain modification approaches, including random mutagenesis (using physical or chemical agents), protoplast fusion, and adaptive evolution, have been widely investigated. These classical strain improvements are random processes wherein it is not possible to predict which type of mutations would arise. Random mutations are introduced into the genome of the strain of interest, followed by a screening and selecting steps in an attempt to obtain strains with desired characteristics (Saxena 2015). Joshi et al. (2010) induced mutations in *Lactobacillus lactis* using classical physical mutagenesis to improve D-lactic acid production from hydrolyzed cane and molasses sugars. Repeated UV-irradiation exposure was able to generate a mutant capable of producing 110 g/L D-lactic acid with 98% of optical purity from 150 g/L of sucrose from hydrolyzed cane sugar in shake flask culture. In another study, a low-pH tolerant mutant of *Lactobacillus delbrueckii*, previously obtained by chemical mutagenesis using nitrous acid, was subjected to genome shuffling strategy through protoplast fusion. After three rounds of genome recombination between this mutant and an amylase-producing *Bacillus amyloliquefaciens*,

the resulting mutant produced 40 g/L of lactic acid from 83 g/L of liquefied cassava bagasse (starch content 50%, w/w), a non-food and low-cost feedstock, with minimal addition of nutrients (only 0.2% of yeast extract and peptone) and low concentration of neutralizing agent (2% $CaCO_3$) (John et al. 2008).

Adaptive Laboratory Evolution (ALE) has become another powerful and non-genetic engineering tool to facilitate and streamline industrial microbial development. ALE consists of adapting cells in a chosen environment with a selection pressure for a prolonged period. After hundreds or thousands of generations, it is possible to naturally obtain mutant strains with desired phenotypes, such as increased product yield/titer, growth rate or substrate utilization, and stress tolerance to pH, temperature or inhibitors (Choi et al. 2019; Cubas-Cano et al. 2019; Sandberg et al. 2019). To improve L-lactic acid production from agro-industrial wastes based on potato stillage and sugar beet molasses, Mladenović et al. (2019) performed ALE of *Lactobacillus paracasei* NRRL B-4564. The first phase of the adaptation was conducted under sequential batch cultivations for 15 days by increasing gradually the concentration of molasses in the medium from 5 to 25%. After another 15 days of adaptation under fed-batch culture, a resulting mutant was able to produce 170 g/L of lactic acid, which was 59% higher compared to parental strain.

Although powerful, classical strain improvement technologies require considerable time for downstream screening and selection, and, when applied alone, can generate an increased number of mutants, containing mostly unimproved strains (Zhang et al. 2018). In this context, recent advances in rational genetic modifications tools have significantly accelerated the development of industrially competitive microorganisms. Over the past three decades, metabolic engineering approaches are being widely used to introduce rationally directed genetic changes into various microorganisms, including those less studied ones, using recombinant DNA technology. These genetic modifications, including eliminating unwanted biochemical reactions, increasing the activity of specific genes, and/or introducing new genes to enhance or redirect metabolic flow in the desired direction, have allowed the construction of customized chassis for the bio-products and bioprocess of interest.

Several studies are evaluating suitable metabolic engineering strategies to improve lactic acid production. Lee et al. (2017) focused on enhancing L-lactate production by *Kluyveromyces marxianus*, an emerging non-conventional yeast with various phenotypes of industrial interest, such as a fast growth rate, various stress tolerance, and wide substrate use. Promising L-lactate dehydrogenases, enzyme responsible for converting pyruvate to L-lactic acid, from heterologous sources and with distinct pH optimums were identified and introduced into *K. marxianus.* A strain co-expressing two L-lactate dehydrogenases simultaneously (one enzyme with an optimum pH of 5.6 and other of 5.3) was able to produce 16.0 g/L of lactic acid with a yield of 0.32 g/g glucose without pH control, whereas the strains expressing those enzymes individually produced a maximum of 8.4 g/L of lactic acid. In another study, *Lactobacillus plantarum* was engineered to increase the optical purity of L-lactic acid during fermentations using raw corn starch (Okano et al. 2018). After deleting simultaneously, the D-lactate dehydrogenase gene and the lactate racemase operon (which catalyzes the interconversion between D-lactate

and L-lactate) and introducing an α-amylase-secreting plasmid to catalyze the hydrolysis of starch, the resulting strain could produce L-lactic acid with a high titer (50.3 g/L), yield (0.91 g/g), and optical purity (98.6%) using raw corn starch as renewable feedstock.

Combined approaches of classical mutagenesis and rational metabolic engineering have also been employed successfully to create industrial strains more efficiently. For the low-pH production of L-lactic acid from both glucose and xylose, Qiu et al. (2018) reconstructed the pentose phosphate pathway for xylose assimilation by introducing four heterologous genes encoding transketolase (tkt), transaldolase (tal), xylose isomerase (xylA) and xylulokinase (xylB) into the *Pediococcus acidilactici* chromosome. Additionally, the endogenous genes phosphoketolase (pkt) and acetate kinase (AckA2) were knocked out to decrease the flux through acetic acid production. The engineered *P. acidilactici* was able to produce 9.8 g/L of L-lactic from xylose. Subsequently, ALE was also applied to accelerate the xylose assimilation rate and increase the L-lactic acid yield. After 66 days, the resulting strain showed about threefold higher L-lactic acid production from xylose.

As described, there are several classical and innovative approaches to develop robust and efficient strains for the biotechnology industry. The choices and results of the upstream process significantly impact the next fermentation and purification developments and scaling. In this context, the integration of processes and teams is crucial for the successful development of industrial bioprocesses based on waste feedstocks.

41.3 Bioprocess Midstream—Fermentation

The fermentation process involves the cell growth to obtain the product of interest, which can be the cell itself or a bio-product produced by it. The bioreactor is the tool used in fermentation process that allows to create environmental conditions to maximize cell and product production. In the bioreactor, parameters such as pH, temperature, agitation, aeration, and dissolved oxygen can be monitored and controlled.

The fermentation can be performed by batch, fed-batch, or continuous mode. In batch process, after culture medium and viable cell inoculation, nothing is added or removed to the culture, except air or another gas, antifoam and acid or base to pH control. Usually, the concentration of nutrients, cells and product vary with process time. In fed-batch fermentation, besides the addition of gas, antifoam and acid or base to pH control, feed can be carried out with one or more nutrients. The feed medium can be continuous or intermittent (pulse-feeding), and the rate can be constant or vary with time. The low concentration of the nutrients supply can minimize the shift in microbial metabolism and/or the inhibition effect by-product or substrate and consequently enhance yield and productivity. In continuous fermentation, after a batch phase period, the culture medium is fed to the bioreactor.

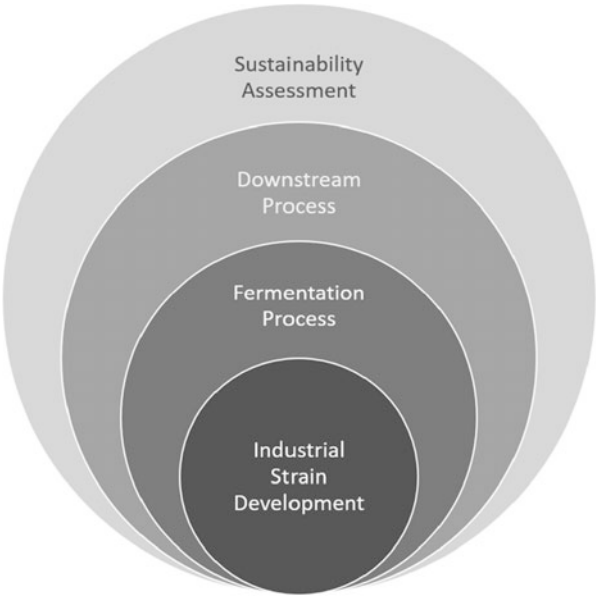

Fig. 41.1 Interactions between the biotech process development teams

The reaction volume in the bioreactor is maintained constant by continuously removal of the fermented broth. It can be performed by the chemostat method, with a limiting substance, or by the turbidostat method, with constant cell mass. Continuous fermentation allows productivity improvement, however, keep the sterile operation is a challenge (Bailey and Ollis 2018; Shuler and Kargi 1992).

The final objective of the fermentation process is to produce an economically and sustainable industrial bioprocess. For this purpose, it must pass through the process development at bench scale, identification of the operational challenges at pilot scale and scale-up to industrial scale.

The development and scaling of the fermentation process involve a constant communication exchange with developers (Fig. 41.1) of the strain, downstream process (DSP) and sustainability assessment (technological, economic and ecosystem analysis), which must become part of routine of the process development team. The main objective is to develop and scale a process to promote metabolic and physiologic conditions to maximize cell and product production.

The strain must be stable from the cell bank, through the inoculum train, until the production scale (Thiry and Cingolani 2002). In the metabolism, the stress can induce metabolic shift, which can result in misincorporation of amino acids in both native and recombinant proteins, impacting the product quality (Fenton et al. 1997; Schmidt 2005).

Culture media are an important environmental factor for microbial metabolism and strongly impact the efficiency of a bioprocess. These cultures can be classified as defined or complex. The carbon source is an important component of the medium. Complex media, composed of nutrients such as yeast extract and tryptone, can be

easily prepared and results in fast cell grow, however, it has a variable composition and variation between batches can occur. On the other hand, defined media has a chemical composition well known, which can be reproducible and facilitate DSP for secreted products (Thiry and Cingolani 2002).

Other important fermentation process parameters are agitation, aeration, pH, temperature, dissolved oxygen, and pressure. Agitation and aeration supply microorganisms with oxygen and promote mix of the broth to obtain uniform suspension and an accelerated mass transfer rate of the metabolic product. Bioreactors with mechanical agitation break the air bubbles and intensify the turbulence of the liquid. The pH and temperature are important parameters for cell growth, but also to stability of the bio-products. Oxygen supply for microbial cultures is often a limiting factor for aerobic microorganisms since oxygen has a low solubility in liquid medium. Dissolved oxygen levels can be increased by supplying pure oxygen (highly expensive and dangerous at large scale) or increasing total air pressure in the bioreactor (Aiba et al. 1973).

In the fermentative process scale-up, it is supposed to transfer the data obtained in laboratory and pilot plant to industrial scale. The scale-down can also occur for well-established process, to make improvement of the process, strain and medium.

Scaling usually follows three steps, laboratory, pilot/demo, and industrial plant. For the most part, the laboratory involves shaker flasks and bench bioreactors. More recently, laboratory high throughput for process development and optimization has been used microtiter plates, instrumented shaker flasks, and miniaturized stirred bioreactors (Marques et al. 2010). More knowledge of the interactions between fluid dynamics and cell physiology in a heterogeneous environment has to receive performed by computational fluid dynamics (CFD), metabolic flux analysis and agent-based modeling (Delvigne et al. 2017).

The pilot plant is indispensable to confirm the improvements of the strain and the medium before going to industrial scale. A instrumented pilot plant can provide valuable physical and metabolic data for a rational scale-up or scale-down.

The success of the scale-up is not result of a straight-lined transposition of experimental data, requiring improvement on each scale. The main relevant physiologically parameters are substrate concentration, biomass, cell viability, metabolites, products, pH, temperature, partial oxygen pressure (pO_2), partial carbon dioxide pressure (pCO_2), and exhaust gas composition. The physical parameters employed for scale-up are mainly those which affect mixing, heat transportation and oxygen supply, such as power input, aeration and agitation rate, heat transfer coefficients, pO_2, and oxygen mass transfer coefficient (kLa). The physical parameters can be combined one each other, or with other variables, in dimensionless numbers that are kept constant, as scaling criterion (Marques et al. 2010; Najafpour 2006; Schmidt 2005).

The dimensionless kLa is the most used physical variable for fermentation scale-up, since it includes parameters that influence oxygen supply. Volumetric power consumption, constant Reynolds, constant impeller tip speed and equal mixing and recirculation time, are also occasionally employed (Garcia-Ochoa and Gomez 2005; Najafpour 2006). Another simple and common scaling method is variation of the

stirrer speed and aeration rate in function of the maintenance of the constant pO_2 (Schmidt 2005).

Lactic acid production via fermentation is the process most widely used by industry worldwide. Microbial lactic acid production has several advantages when compared to chemical route, such relatively lower temperatures, lower energy consumption, and high purity (Oliveira et al. 2018). Industrial microbial production of lactic acid is still predominantly performed with carbohydrates such as glucose, lactose, starch, and sucrose from sugar beet, molasse, and whey. However, in recent years, several laboratory-scale fermentation studies have aimed to evaluate different renewable materials, as well as to validate and improve the performance of the lactic acid-producing strains and find the optimal production conditions in order to develop more sustainable and economically viable bioprocesses for large-scale lactic acid production.

Batch mode fermentation is typically used for industrial production as it can allow higher lactic acid titers with reduced risk of contamination despite the disadvantage of generally resulting in lower productivities (Ahmad et al. 2020; Ghaffar et al. 2014; Rawoof et al. 2021). However, continuous and fed-batch modes, with initial high cell density culture or cell recycle have also been reported. Simple batch and repeated-batch processes with cell recycle were evaluated for L-lactic acid production using wild-type *Enterococcus faecalis* and hydrol (an efficient carbon source derived from glucose production), soybean curd residues (a potential nitrogen source derived from soybean products), and malt as renewable substrates (Reddy et al. 2016). Ten repeated batches with cell recycle were performed at 38 °C and pH 7.0 controlled automatically by the addition of NaOH, with a total fermentation time of 200 h. Biomass concentration increased during repeated-batch fermentations, reaching 26.3 g_{DCW}/L, removing inoculum preparation step at each fermentation. The productivity of L-lactic acid also increased significantly from 3.20 to 6.37 g/L·h, indicating that repeated-batch fermentations with cell recycle are an efficient bioprocess for industrial production of lactic acid. Carpinelli Macedo et al. (2020) evaluated the production of lactic acid by *Lactobacillus amylovorus* using hydrolyzed cassava bagasse and corn steep liquor as renewable feedstocks. Batch without pH control and fed-batch with pH controlled automatically at 6 using NaOH strategies were carried out at 37 °C. The maximum lactic acid production and productivity under batch mode were 31.6 g/L and 0.11 g/L·h, respectively. In contrast, higher titer (66.9 g/L) and volumetric productivity (0.46) were achieved when controlled fed and pH maintenance were performed.

Many bioprocess variables, as temperature, pH, neutralizing agent, aeration level, substrate concentration, inoculation size, sterilization, have been adapted and optimized for improving lactic acid production. The definition of these variables depends on the type of raw material, chassis, expected yield/titer or purity. The effects of critical parameters, such as temperature (25, 30, 35 and 40 °C), inoculum size (5, 10, 15 and 20%), and sugar concentration (8, 13, 18, and 35 g/L of reducing sugar concentration) were examined separately during batch fermentations of *Lactobacillus casei* using soybean straw hydrolysate for L-lactic acid production (Wang et al. 2014). The maximum L-lactic acid yield and productivity were obtained with

the higher initial reducing sugar concentration (35 g/L) and intermediate conditions for temperature (30 °C) and inoculum size (10%). Other critical parameter in bioprocess is pH, mainly during acid organic production. pH is closely related to the enzymes involved in the metabolism process and cells nutrients transport, and changes in pH can affect microbial activity and the efficiency and final titers of the bio-product. Furthermore, as mentioned earlier, the choice of neutralizing agent for pH control is pivotal for a sustainable bioprocess. In this context, Liu et al. (2014) studied the effects of KOH, $Ca(OH)_2$ and NH_4OH as neutralizing agent for D-lactic acid production by a genetically engineered *Escherichia coli* strain. Fermentation neutralized by $Ca(OH)_2$ achieved a volumetric productivity three times higher in addition to a slightly higher yield of D-lactic acid compared to that achieved by KOH or NH_4OH. According to the authors, $Ca(OH)_2$ is the cheapest neutralization agent compared to the other two in the Chinese market, which makes it a potential base for industrial production of D-lactic acid. Nakano and co-workers compared the employment of $Ca(OH)_2$, NH_4OH, and NaOH as neutralizing agents for lactic acid fermentation and evaluated its impact on lactic acid recovery. In Simultaneous Saccharification and Fermentation (SSF) process with *Lactobacillus delbrueckii*, $Ca(OH)_2$ as neutralizing agent resulted in higher lactic acid productivities (3.59 g/L) compared with NH_4OH (1.51 g/L) and NaOH (1.4 g/L). The molarity of the lactate in the fermentation broth was reduced using calcium hydroxide, besides that, it suggests that the divalent cation (Ca^{2+}) was more effective in neutralizing cultures compared with monovalent cations (Na^+ and NH_3^+) (Nakano et al. 2012).

The choice of nitrogen source in the culture medium also plays a key role in the development of economically viable bio-based processes. Balakrishnan et al. (2020) evaluated the production of D-lactic acid by *Lactobacillus delbrueckii* using the low-cost Kodo millet bran residue, an abundant grain in India, Africa, and China, with significant amount of starch, protein, and other essential nutrients. The effects of several types of nitrogen supplements (yeast extract, beef extract, bacteriological peptone, brain heart infusion, soy peptone, whey protein hydrolysate, casein enzyme hydrolysate, urea, and sodium nitrate) and its optimized dosage on D-lactic acid production was studied. The results indicated that casein enzyme hydrolysate is the most suitable low-cost nitrogen source and that increased dosage of this nitrogen source has a positive effect on LA production. However, no significant increase in specific growth rate and D-lactic acid productivity was observed in fermentations with casein enzyme hydrolysate concentration above 5 g/L. Therefore, an optimum casein enzyme hydrolysate dosage of 5 g/L should be considered for a more economical bioprocess.

As described above, there are different approaches for fermentation development and scaling process. For each process, for each bio-product, there is a different strategy that can be elaborated. The process must be well characterized, and all variables that impact the product yield and quality must be known. In most cases, the scaling will not be a result of a conclusive and straight-lined experimental data, but rather a result of an accurate analysis of the experimenter, which depends on their experience, ability and intuition (Marques et al. 2010; Schmidt 2005). The interaction with other developers must be frequent, since the strain development, the

fermentation process, downstream process, and sustainability assessment work like a chain, and are strongly linked.

41.4 Bioprocess Downstream—Separations and Purification

Downstream process (DSP) is an important pillar of the industrial bioprocess to obtain a purified bio-product. It follows a sequence of unit operation, which usually consists of initial recovery, purification, and polishing.

In the initial recovery, there are a cell separation from the broth, step performed mainly by centrifugation, filtration, or flotation. For intracellular bio-products (Fig. 41.2), the cells must be broken, and the cell debris removed. The cell lysis could be performed by operations such as high-pressure homogenizer, mill, or an enzymatic method. The clarification obtained from cell homogenate can follow steps such as concentration, purification, or formulation. Already for extracellular bio-products (Fig. 41.2), the clarified broth can be concentrated by precipitation or ultrafiltration, followed by purification and formulation (Harrison et al. 2015; Roque et al. 2004; Sutherland and Chase 2011). Therefore, DSP usually entails several unit operations to obtain the product to the required specification. However, it is not just about to achieve the required purity, the DSP must be robust, reliable, and scalable.

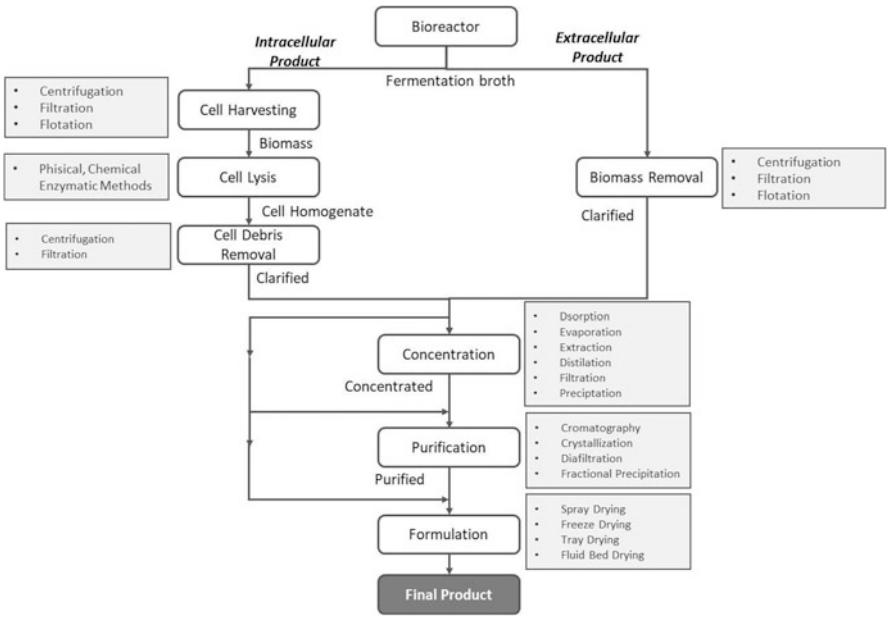

Fig. 41.2 General steps and operations involved in a bio-product purification

For that, essential parameters such as purity and recovery yield must be monitored (Rosa et al. 2010).

In this section, we will focus on lactic acid purification produced by fermentative route. Indeed, depending on the application of the lactic acid, a specific purity is required: industrial grades 88–90%; food grades 25–90%; pharmaceutical and cosmetic grades 90%, and specialty grades 80–98%. Therefore, a set of DSP steps are necessary to achieve the purity grade (Komesu et al. 2017b).

After fermentation process is generated, a whole broth composed by cells, residual of sugars, components from previous steps (pretreatment and hydrolysis of lignocellulosic materials) to obtain fermentable sugars, salts, media components, by-products, besides the lactic acid, which demand multistep for lactic acid recuperation and purification. Downstream process is a key element to obtain cost-effective production of lactic acid in high purity, since the DSP is estimated for over than 50% of the production cost (Datta and Henry 2006; Kumar et al. 2020; Pal et al. 2009).

The fermentation for acid lactic production is carried out at near-neutral pH, hence, during the neutralizing process, it produce salt instead of acid, which is a challenge for purification. A traditional process applied for lactic acid recovery is precipitation, which is detailed below.

41.4.1 Precipitation

Precipitation process is an operation downstream, which consists of an obtention of solid from a solution. This step is usually carried out in the first stages of purification. The precipitation process allows to reduce the reaction volume by precipitation of the interest product and its resuspension in a smaller volume. Also, it can be used to purify through the fractional precipitation of the interest product, leaving the contaminating in the mother solution. This technique is relatively inexpensive, can be carried out continuously, and with simple equipment (Harrison et al. 2015).

During fermentation, lactic acid is produced and accumulated in the broth, decreasing the pH, which is inhibitory to the cell. In the traditional microbial lactic acid production, calcium carbonate ($CaCO_3$) or calcium hydroxide ($Ca(OH)_2$) are usually employed to maintain the pH control around 5–7 producing calcium lactate salt. After fermentation, the broth is treated with sulfuric acid to convert the calcium lactate into lactic acid and calcium sulfate ($CaSO_4$), followed by filtration to obtain free organic acid. Then, the filtrate is evaporated to recover the lactic acid, which achieve technical grade between 22 and 44% (Fig. 41.3) (Datta and Henry 2006; Komesu et al. 2017a). For high purity (Fig. 41.3), it is required additional steps of esterification with ethanol or methanol, distillation to recover the ester, hydrolysis with water and evaporation to recycle the alcohol and obtain the pure acid lactic (Datta and Henry 2006).

Despite precipitation be a simple technique, it consumes high quantity of sulfuric acid and produces a huge quantity of calcium sulfate as a solid low-cost waste

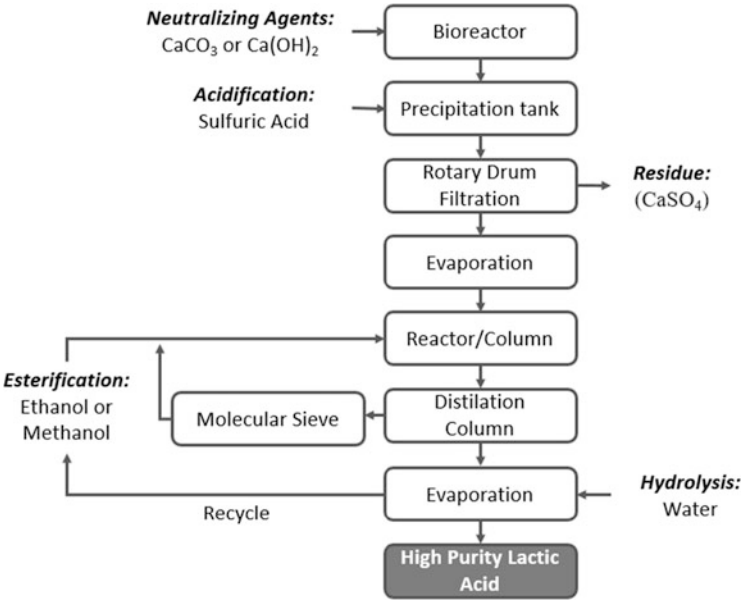

Fig. 41.3 General sequence of lactic acid recovery by precipitation

residue. Besides that, low purity of lactic acid often is achieved, demanding other steps to increase its purity (Kumar et al. 2020; Pal et al. 2009).

Approaches to improve the efficiency of lactic acid precipitation have been carried out. Kwak et al. (2012) proposed a precipitation process to recover alkyl lactate from ammonium lactate. Recovery of lactic acid from ammonium lactate solution by acidification with sulfuric acid is a difficult task, since ammonium sulfate [$(NH_4)_2SO_4$] has high solubility and lack of precipitation. The addition of methanol during the acidification of ammonium lactate decreases the solubility of the $(NH_4)_2SO_4$, which could be separated by filtration. The clarified with lactic acid was transformed into methyl lactate by an esterification reaction with methanol, which could be separated by distillation. The processes can be performed at room temperature with simple equipment, and the residual ammonium sulfate can be used to produce ammonia and sulfuric acid or be sold as low-cost fertilizer.

A patent issued to ZeaChem Inc, an alternative bioprocess using $CaCO_3$ as a neutralizing agent during fermentation and nitric acid as precipitation agent is described. This strategy allows lactic acid recuperation and concomitant production of ammonium nitrate processed as nitrogen fertilizer and $CaCO_3$ can be recycled in the process (Verser and Eggeman 2006).

41.4.2 Membrane Process Separation

An alternative lactic acid purification process is based on membrane separation, detailed below. Filtration process is an operation of downstream, which consists in a separation of a particulate from a suspension, according to their size, by flowing under a pressure differential. The filtration process can be conventional, where the fluid flows perpendicular to the filter element, or crossflow filtration, where the fluid flows parallel to the filter element to minimize buildup of solids on the filter. In bioprocess, conventional filtration is used for sterile filtration and for extracellular products, where the conventional filtration is employed to retain the cells in cake (solid phase) to obtain the product in the clarified phase (liquid). Crossflow filtration is used for separation of cells and its components, concentration and for exchange and remove of salts. A membrane process can achieve high levels of separation and purification and can be integrated into other operations such as bioreactors, eliminating the separation step in a compact design (Harrison et al. 2015; Pal et al. 2009).

In lactic acid production, the decrease of the pH is a bottleneck that can result in a reduction of the productivity. Membrane-coupled continuous fermentations (Fig. 41.4) have been carried out in order to remove the lactic acid produced (maintaining pH desired for fermentation) and ensure high cell concentration and productivity (Pal et al. 2009). Membranes of microfiltration, ultrafiltration, nanofiltration, reverse osmosis and electrodialysis can be employed to separate fermentation products from lactic acid produced. Depending on membrane porous size, different fermentation products are separated. Crossflow microfiltration retains only cells, while the permeate removes unconverted carbon sources, nutrients, proteins, salts, water, and lactic acid. Differently, crossflow ultrafiltration, retains cells and proteins, while removes unconverted carbon sources, nutrients, salts, water, and

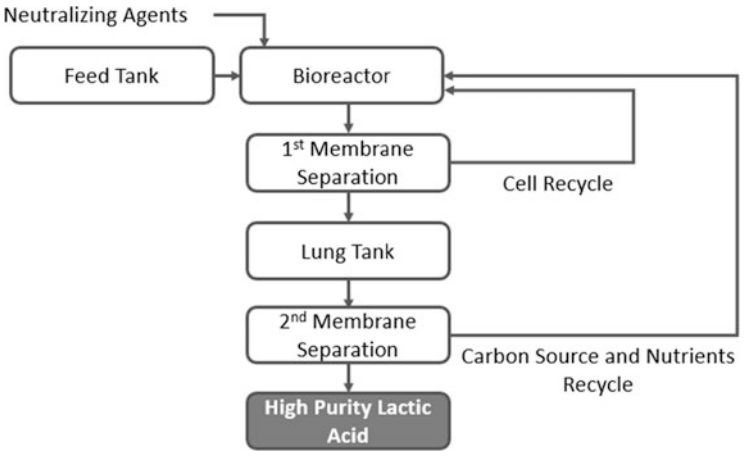

Fig. 41.4 Integrated continuous fermentation with membrane process in two stages. First stage—microfiltration or ultrafiltration membrane; second stage—nanofiltration or reverse osmosis

lactic acid. However, the permeate flux can be decreased due to membrane clogging, fouling and concentration polarization (Crespo et al. 1992; Diosady et al. 2005). The use of microfiltration is recommended before the ultrafiltration to increase the ultrafiltration efficiency, avoiding the fouling of the membrane by high molecular weight protein. Indeed, the fermentation with cell recycle by microfiltration or ultrafiltration results in a culture with high cell density and consequently in an increase of the viscosity and lowering of permeate flux, which can be overcome by cell bleeding (Crespo et al. 1992; Diosady et al. 2005).

Nanofiltration membrane can retain cells, unconverted carbon sources, nutrients, proteins, and salts, while removing water and lactic acid. The membrane process coupled in a bioreactor in two stages, first microfiltration followed by flat sheet crossflow nanofiltration as second step, can achieve monomer grade lactic acid with high productivity (Fig. 41.4) (Pal et al. 2009). US Patent employed a ceramic tubular ultrafiltration in the first stage for cell separation and a nanofiltration in the second stage, resulting in a long-term operation (Russo and Kim 1996).

In reverse osmosis, the separation is based on solution diffusion mechanism, which demands a high operating pressure. Reverse osmosis, as well as nanofiltration, retains cells, unconverted carbon sources, nutrients, proteins and salts, while removes water and lactic acid. In both strategies, unconverted carbon sources and nutrients can recycle in the bioreactor, while allow the lactic acid purification (Pal et al. 2009).

In electrodialysis (ED) the separation is based on the electromigration of ions through a stack of cation and anion exchange membranes. Applying an electric potential between the electrodes, cations migrate to cathode and anions to the anode (Pal et al. 2009). Electrodialysis is applied to remove salts from solutions or to concentrate ionic substances. It involves two stages: first, monopolar electrodialysis (MEP) separates and concentrates the lactate salt from the fermentation broth, while in the second stage, the bipolar electrodialysis (BED) converts the lactate salt into lactic acid (Hábová et al. 2004). For electrodialysis efficiency, it is necessary a cell-free broth, therefore, studies have been performed with microfiltration, ultrafiltration or nanofiltration prior to electrodialysis (Bouchoux et al. 2005).

Membrane-based technologies show high selectivity, resulting in high levels of separation and purification. The integration of the membrane with a fermentation process allows simultaneous production and purification, avoiding additional equipment and reducing equipment investment cost. However, the high cost of membranes, polarization and fouling are still a challenge for the use of these processes.

Other lactic acid separations include liquid–liquid extraction, molecular distillation, and reactive distillation (Datta and Henry 2006).

41.5 Sustainability Assessment

During the stages of development of a new process technology, it is extremely important to assess the sustainability of the process which is being scaled-up. Despite several advantages are being reported for the biochemicals production, not all biochemicals are consistently more sustainable than equivalent petrochemicals. This stresses the need for metrics to evaluate the new process's sustainability to guide the research decisions during the development.

The three key principles of sustainable development are economic, environmental, and social aspects. This section covers methodologies to assess the economic and environmental aspects during the scaling-up stage of the project. Social principal is not less important, but still a challenge in terms of methodology.

In typical industrial biotechnology, several decisions are made when designing an industrial plant, and these choices have impacts that need to be evaluated. For instance, during strain optimization, strains are mainly selected based on yield, titer, and productivity. However, the presence of some specific bio-products may lead to higher associated downstream processing costs. Likewise, for proper downstream separation of impurities, extensive use of chemicals or utilities may increase environmental impacts. Very often, the environmental and economic performance implies trade-offs that should be considered and evaluated early in the technology scale-up. An iterative approach considering techno-economic and environmental impacts can optimize the final biochemical process.

Assessing the economic viability of future technologies is part of the product development in biotechnology. This chapter focus on economic assessment during the scale-up stage of a process.

41.5.1 Assessing the Economic Sustainability (Techno-economic Analysis)

The cost estimation methodology for the scale-up stages of biochemical development is similar to the methodologies applied to estimate the cost of the industrial plant. Here it is important to stress the main goal of the process scale-up is to design a complete process, from the feedstock to the final product for a specific application and to decrease the technical and economic risks for the investment. The cost of the final industrial plant must be assessed as well as the cost of the scale-up step, which includes the cost of operation of a pilot or demo plant.

The economic feasibility of any new process depends on the overall yields and costs associated with the production process. The costs can be classified as capital and operational costs. Capital costs are related to acquisition of the required equipment, automation, infrastructure, buildings, engineering and construction and contingencies, and others, that are required to build an industrial unit. The operational costs refer to the expenses to run the process and are proportional to the plant output

or operation rate. It includes raw materials, utilities, consumables (chemicals and catalysts), effluent disposal, packing, maintenance, and labor.

Specifically for the scale-up steps, it must be considered if there is an existing pilot plant facility. In this case, scale-up planning should first consider whether a retrofit would be necessary and must estimate the related costs of it. Secondly, the scale-up should establish the experimental program and schedule and estimate the operation costs related to this plan. The former mentioned costs related to the equipment are Capital costs (CAPEX), while the costs related to the operation of the facility are the operational costs (OPEX).

A pilot plant is a collection of equipment designed and constructed to demonstrate technical feasibility of a new process and its performance.

In some cases, a company may desire to invest in his own new multipurpose facility to have a research installation where the innovation project portfolio can be proven. This is not the general case once high investments are necessary to build and erect a new installation.

However, most of the time it is more advantageous to contract services of a pilot plant platform and the scale-up development team. There are some examples of facilities in the world dedicated to the development of new processes, such as BBEU (Bio Base Europe Pilot Plant), CBP Fraunhofer, BPF (Bio-based Process Pilot) and Pilot Plant at LNBR/CNPEM [Brazilian Biorenewables National Laboratory (LNBR), part of the Brazilian Center for Research in Energy and Materials (CNPEM)].

41.5.1.1 Capital Cost (CAPEX)

Cost estimation strategies for pilot or demonstration plants are similar to the costing of the industrial facility, however, the small scale of these facilities implies more error, and final estimate can have lower accuracy. The installation costs, construction, labor and overhead represent a larger percentage in pilot and demo plants.

There are three basic methods for estimating the costs of a new pilot plant: similarity, cost ratios and detailed labor and materials (Palluzi 1991)

- Similarity involves estimating the cost of design and construction of the new pilot plant based on a similar unit. It is a fast method, nevertheless, it has low accuracy with errors of $\pm 100\%$. It can be used as an order of magnitude at very early stages of the research when little piece of information about pilot plant cost is available.
- Cost ratios involve estimating costs by relating the overall cost of the pilot plant or part of the pilot plant to a known factor such as the cost of main equipment, the number of control loops, the size of the equipment, or a variety of similar factors. The cost is estimated by using the ratios to develop the cost of the entire unit or of some subsystems. Although cost ratios are a widespread methodology for plant-estimating tool, the cost ratios are rarely available for pilot plant equipment. Typically, the accuracy is ± 25–50%.

- Detailed labor and materials estimation involves breaking the pilot plant construction down into a detailed series of small tasks and estimating the labor and materials required for each separate task. This method has accuracies of ±10–20% but requires more effort than the previous methodologies.

A good practice is to use similarity or cost ratios estimation methods at the early stages of the research when low accuracy is accepted. Detailed cost ratios or detailed labor and materials estimates are generally developed prior to appropriation of funds to have a more accurate estimate for budgeting and cost control (Palluzi 1991).

The reduction of the time involved between the beginning of the pilot plant project and the data generation is always a concern. An average time of 6–18 months to progress through this process (3–12 months for design, 3–12 months for construction and 1–6 months for commissioning and start-up). This time can be decreased if careful and detailed planning is done.

41.5.1.2 Operational Cost

The costs for operating a pilot or a demo plant are a summary of the feedstock costs, product disposal, utilities, operating labor, spare parts, maintenance, and support services during the planned timeline for scaling. To have a forecast of the operation cost, some preliminary mass and energy balances must be done based on first assumptions and process basic design. Modeling tools using available commercial simulators or simple excel datasheets may be used to have a first figure of the mass and energy balances.

41.5.2 Assessing the Environmental Sustainability

The environmental sustainability of biochemicals is becoming increasingly important. During the stages of the technology development, environmental impacts can be preliminary assessed by tools such as Life Cycle Assessment (LCA). As the project advances, the performance indicators of the process are continually updated, and the initial environmental assessment tools should be periodically revised.

LCA is an import tool to compare environmental impacts of bio-based to traditional fossil products, and it is important to emphasize that the fact of using a renewable feedstock and a biotechnological process is not a guarantee that the product has less environmental impacts. Green routes use renewable feedstocks, but often generate very low product concentration, which needs to be purified. If the process design is not carefully done using heat thermal integration and water recycle, final process may not be more environmentally friendly when compared to the traditional route. This reinforces the need for LCA assessment during the research to guide design selection to have a good trade-off between economic and environmental aspects.

41.5.3 Managing Risks

The scale-up step is the most potentially risky of all phases of new process development. Some of the risks faced during this step are described here, as well as some recommendations of how to mitigate them:

- *Technical Risks*

The use of unproven technology in scale-up is an unavoidable risk, as very often an innovative technology is required. Because of this inherent risk, the scale-up design should be conducted in a way to minimize the risk of using unproven technology or have a mitigation alternative. The designer should, whenever possible, use technology that has been proved through use in commercial facilities or should consider it as a possible mitigation plan.

The integration of multiple new technologies tends the system to more complexity. If possible, the design of the process should be simple. The use of modular scale-up equipment and flexibility of having other candidates of unit operations that can substitute the main design is also recommended.

- *Operational Risks*

It is highly recommended to have a plain for the scale-up program as well to set a training program for the operational team. Sometimes, if some disturbances of the process are expected, it should be investigated at lab scale to prepare a "what to do" plain to mitigate and inform the operation team. Biotechnological processes face risks of contamination, and the septic control is an important consideration for the design and for the elaboration of operation procedures.

- *Regulatory Risks*

The scale-up must consider a variety of regulation standards related to govern workplace safety and waste disposal. Compliance with regulation must be thought from the earliest stages because of the potential costs involved. Waste disposal regulation, safety at workplace regulation, product regulation, GMO regulation must comply.

Finally, to conclude this topic, it is worthwhile to stress that a successful scale-up does not guarantee successful commercial plant operation, however, it considerably decreases the risk.

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