Current Trends and Prospects in Antimicrobial Peptide Bioprocessing

Kamila Botelho Sampaio de Oliveira, Michel Lopes Leite, Gisele Regina Rodrigues, Nicolau Brito da Cunha, Simoni Campos Dias, and Octavio Luiz Franco

Contents

1 Introduction 110
2 Antimicrobial Peptides 112
2.1 Properties 112
2.2 Mechanisms of Action and Targets 113
2.3 Classification 115
3 Upstream Process Development 115
3.1 Recombinant AMP Production in Microbial Cells 117
3.2 Strategies to Enhance the Heterologous Expression Level 119
4 Scale-Up from Small- to Large-Scale Fermentation 124
4.1 Batch Processes 125
4.2 Fed-Batch Processes 126
4.3 Continuous Processes 128

K. B. S. de Oliveira · G. R. Rodrigues · N. B. da Cunha
Centro de Análises Proteômicas e Bioquímicas, Pós-graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, Brazil

M. L. Leite
Centro de Análises Proteômicas e Bioquímicas, Pós-graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, Brazil

Departamento de Biologia Molecular, Instituto de Ciências Biológicas, Campus Darcy Ribeiro, Universidade de Brasília, Brasília, Distrito Federal, Brazil

S. C. Dias
Centro de Análises Proteômicas e Bioquímicas, Pós-graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, Brazil

Universidade de Brasília, Pós-graduação em Biologia Animal, Campus Darcy Ribeiro, Brasília, Brazil

O. L. Franco (✉)
Centro de Análises Proteômicas e Bioquímicas, Pós-graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, Brazil

Universidade de Brasília, Pós-graduação em Patologia Molecular, Campus Darcy Ribeiro, Brasília, Brazil

S-Inova Biotech, Pós-graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande, Mato Grosso do Sul, Brazil

B. H. A. Rehm, D. Wibowo (eds.), *Microbial Production of High-Value Products*, Microbiology Monographs 37, https://doi.org/10.1007/978-3-031-06600-9_5

5 Purification of AMPs: Downstream Process Development 129
5.1 Recovery 129
5.2 Purification 130
6 Optimization of the Industrial Processes 131
7 Conclusions and Future Directions 132
References 133

Abstract The increase in resistance to conventional antimicrobials in recent years has boosted the search for new antibiotics to treat serious infectious diseases, especially those generated by multi-resistant bacteria. In this context, antimicrobial peptides (AMPs) are alternative molecules for use as new therapeutic agents. AMPs are small bioactive proteins commonly produced by all living organisms, and they can be part of innate immunity. Due to their broad-spectrum antibacterial potential and other activities, including immunomodulatory and antitumor, they are of great interest to the pharmaceutical industry's production of biopharmaceuticals. Among the technological platforms applied in the process of development and manufacturing of AMPs, recombinant DNA technology has enabled the production of such molecules using bacterial and yeast cells as expression host systems on a laboratory scale and in large-scale environments. Furthermore, different bioprocessing strategies can be used for peptide industrial production, aiming to optimize the yield, make cultures more robust and significantly increase cell density. In this chapter, we will address recent developments and future directions in AMPs bioprocessing, including microbial expression systems, as well as bioprocessing and purification technologies. Here we also describe successful cases in this field and emphasize the prospects and challenges related to AMPs bioengineering.

1 Introduction

The increase in microbial resistance to antibiotics is a major public health problem around the world. Methicillin-resistant *Staphylococcus aureus* (MRSA) and β-lactamase-resistant *Escherichia coli* (ESBL) are bacterial strains that recur in many hospitals (Assis et al. 2017; Wang et al. 2019), mainly infecting patients whose immune system is compromised by other diseases or invasive therapies (Leite et al. 2019). Bacteria such as *Pseudomonas aeruginosa, Enterococcus faecium*, *Acinetobacter baumannii*, *Klebsiella pneumoniae,* and *Enterobacter* species are also commonly responsible for nosocomial infections exhibiting high microbial resistance to currently available drugs (Laws et al. 2019).

The emergence of multi-resistant microorganisms such as those mentioned above may occur due to the excessive and/or incorrect use of antimicrobial drugs by humans and other animals (Lombardi et al. 2019; Bhopale 2020; Dijksteel et al. 2021). As a result, many drugs commonly used to fight those pathogens are no longer effective (Leite et al. 2019). About 700,000 people die annually from resistant

bacterial infections. If measures are not taken to change the current scenario, it is estimated that by 2050 the number of deaths will be ten million per year (Mishra et al. 2017; Laws et al. 2019). The development of new, broad-spectrum, and low-toxicity drugs as an alternative therapy to conventional antibiotics is highly desirable. Both academia and industry are working on prospecting candidate molecules and producing new therapies to combat infectious diseases (Andersson et al. 2016; Leite et al. 2019).

In this context, antimicrobial peptides (AMPs) have emerged as a new approach for replacing or complementing traditional therapeutic compounds in combating resistant microorganisms (da Costa et al. 2015; Chen and Lu 2020). They are naturally produced by various living organisms, such as microorganisms, plants, and animals, acting as important immune system components against exogenous pathogens (Wang et al. 2019; Leite et al. 2019). Generally, they present activity against several microorganisms and can perform more than one function in some cases, including acting as an immunomodulator or antitumor, so they are considered promiscuous molecules (Franco 2011; Haney et al. 2017; Leite et al. 2019).

Due to the different functions that they can perform simultaneously, AMPs are molecules that seem to have great potential as candidates for new drugs, especially antibiotics (Tornesello et al. 2020; Liscano et al. 2020). The development of new AMP-based drugs needs these molecules to be obtained in large quantities for biotechnological studies and pharmaceutical evaluation (Parachin et al. 2012). There have been notable improvements in producing AMPs on an industrial scale during the last few years, due to several new technologies (Gupta and Shukla 2017). Innovations in the upstream bioprocessing steps, such as selection and development of the cell line, optimization of cell culture parameters, and the use of feeding strategies, have the ultimate goal of large-scale production (Gronemeyer et al. 2014; Tripathi and Shrivastava 2019). The application of recombinant systems in cell line construction is opportune in terms of yields and costs for AMPs production (Parachin et al. 2012).

Among these recombinant systems, microorganism hosts are generally the most used, because they have characteristics that enable high levels of synthesis, modification, and secretion of heterologous AMPs. Various recombinant proteins are already available on the market, including AMPs produced by these hosts (Gupta and Shukla 2017). *E. coli* bacteria is the most widely used microbial cell factory, since its genetic and biological processes are well known, exhibiting fast growth rate, high yield and simple upstream process (Khow and Suntrarachun 2012; Briand et al. 2016; Kaur et al. 2018). Otherwise, among the yeast systems, *Saccharomyces cerevisiae* is the most established. It is a robust, stress-tolerant yeast and uses simple nutrients (Öztürk et al. 2017).

After developing the expression system, the next step consists of producing the small-scale recombinant protein to screen and select transforming clones. At this point, it is essential to monitor the cultivation conditions, as they directly affect the expression of the recombinant AMP. Optimizing the temperature, pH, aeration, agitation, media composition, the concentration of inducers, induction time and the feeding strategies make the development of bioprocesses more effective (Kaur

et al. 2018). Continuing the process, bioreactor systems and bioprocess strategies such as batch, fed-batch, and continuous culture are employed for the production of large-scale AMPs as biopharmaceuticals (Tripathi and Shrivastava 2019). Subsequently to large-scale production, a key purpose is the recovery of the target biomolecule and removal of the impurities present in the culture medium (Gupta and Shukla 2017).

Known as the downstream process, this phase has innovative technologies for handling large volumes in production processes, as well as for the recovery of the recombinant biomolecule of interest (Gupta and Shukla 2017). Filtration removes cell biomass, providing culture medium clarification, and affinity chromatography captures the recombinant protein using specific separation resins, promoting a high degree of purity for the proteins of interest (Singh et al. 2013; Kimple et al. 2013; Arora et al. 2017). In summary, this chapter addresses innovative approaches to upstream and downstream processes for AMPs yielding biopharmaceuticals. Selection of the proper expression hosts, development of bioprocesses, recent strategies in bioprocessing, techniques related to purification, and ways to achieve lower production costs, while boosting manufacturing flexibility and final product quality, are here described.

2 Antimicrobial Peptides

2.1 Properties

AMPs are small bioactive proteins that are effective against several species of Gram-positive and -negative bacteria, fungi, parasites and viruses (enveloped and non-enveloped) (Bhopale 2020; Moretta et al. 2021). These molecules are also known a host defense peptides (Boto et al. 2018; Liang and Diana 2020), and they are essential elements of the innate immune system of living organisms, acting as a first defense line against microbial actions displaying microbicidal, bacteriostatic, and cytolytic properties (Sinha and Shukla 2019; Moretta et al. 2021). AMPs are the primary defense line against pathogenic microorganisms for plants and insects, as these do not present an adaptive immune system. In bacteria and other microorganisms, AMPs act in defense of their environmental niche (Browne et al. 2020). The bacteria *Paenibacillus polymyxa*, for instance, which develops in plant roots, produces the antibiotic polymyxin, capable of breaking down *P. aeruginosa* or *S. aureus* biofilms (Quinn et al. 2012).

AMPs are evolutionarily conserved molecules that are extremely diverse in composition and length, with different sequences, structures, and sources, but they have some typical features (Bhopale 2020). Most AMPs are generally characterized as short molecules containing fewer than 100 amino acid residues and have molecular masses ranging from 1 to 10 kDa. These peptides commonly possess a positive net charge ranging from +2 to +11, attributed to the presence of positively charged residues such as lysine and arginine residues, thus being characterized as cationic

molecules (Mahlapuu et al. 2020). These molecules usually have a considerable amount of hydrophobic residues (typically 50%) such as valine, leucine, isoleucine, alanine, methionine, phenylalanine, tyrosine, and tryptophan in the peptide sequence (Lee et al. 2017; Thapa et al. 2020; Dijksteel et al. 2021).

2.2 Mechanisms of Action and Targets

Generally, the first AMP interaction with the bacterial cell membrane occurs through hydrophobic and electrostatic interactions between cationic residues and anionic components of the microorganism's membrane (Moravej et al. 2018). The negative microbial surface charge may be provided by phospholipid head groups such as phosphatidylglycerol, cardiolipin, or phosphatidylserine (Boto et al. 2018). Eukaryotic membranes have zwitterionic phospholipids such as phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin. These phospholipids contribute to membrane net charge at physiological pH. In addition to zwitterionic phospholipids, the presence of cholesterol molecules along the membrane may also contribute to a reduction in membrane fluidity and flexibility, which can generally reduce AMP activity (Boto et al. 2018; Dijksteel et al. 2021). Therefore, AMPs are generally non-toxic to mammalian cells, which is an attractive feature for their therapeutic use (Silva et al. 2011; Browne et al. 2020). Although AMPs' mechanism of action is still largely unknown, the electrostatic attraction between negatively charged cells components and positively charged AMPs is understood to be important as a first step, resulting in a strong interaction and further target cell membranes disruption (Tornesello et al. 2020).

After initial electrostatic interactions, self-assembly peptide accumulation on the bacterial membrane surface may cause membrane integrity loss and intracellular component leakage after reaching certain concentrations (Mirski et al. 2017; Dijksteel et al. 2021). Furthermore, there are a several widely accepted models of action that usually involve bacterial cytoplasmic membrane integrity being disrupted in many ways. Among them are the barrel-stave pore, detergent micellization, toroidal pore, disordered toroidal pore, membrane thinning/thickening charged lipid clustering, non-bilayer intermediate formation, oxidized phospholipid targeting, anion carrier, and non-lytic membrane depolarization, among others (Nguyen et al. 2011; Mahlapuu et al. 2020).

In the barrel-stave model, AMPs can self-organize into cylindrical bundles, which insert themselves in a perpendicular way into the membranes. Aqueous pore lumen formation may occur with the orientation of hydrophobic portions towards the hydrophobic bilayer interior. The pore may cause cytomembrane permeabilization, osmotic imbalance and further cell death (Seyfi et al. 2020). In the toroidal model, the pore formation occurs when the AMPs insert themselves perpendicularly into the bacterial plasmatic membranes, a mechanism similar to the barrel-stave model. However, in this model, the nonpolar AMPs amino acid residues interact with lipid head groups, causing membrane deflection and, further, generating torus

pores (Lee et al. 2017; Moretta et al. 2021). Another possibility is the carpet model, in which the AMPs are accumulated and arranged parallel to the cell membrane, covering it completely. At the same time, micelles are formed with the initial ruptured membranes, through the hydrophilic amino acids interactions of polar phospholipid heads, causing cytomembrane disruption (Deng et al. 2017; Lee et al. 2017; Zandsalimi et al. 2020). Moreover, peptides are capable of interacting with several targets, including proteins and carbohydrates, acting not only on the surface, but also inside the cell (Silva et al. 2011; Kumar et al. 2018). In addition to destabilizing bacterial membranes and causing their rupture (membrane-acting peptides), AMPs can also cross the membrane, destabilizing normal cellular processes, such as cell division, protein, nucleic acid and cell wall synthesis, being classified as "non-membrane acting peptides." AMPs can act against pathogens by causing different stresses at the same time until the combined action causes cell death (Boto et al. 2018). AMPs that act through membrane destruction are also able to act through non-destructive membrane, in addition to acting independently or synergistically with other AMPs (Dijksteel et al. 2021).

Nonetheless, AMPs have a therapeutic potential that goes beyond their antimicrobial activity. Some AMPs can also exhibit cytotoxic activity against tumor cells (Wang et al. 2019). Most tumor cells also have a surface negative charge on the membrane due to anionic overexpression of molecules such as phosphatidylserine. This characteristic allows the interaction with cationic AMPs (Wang et al. 2019). AMPs are also capable of inhibiting inflammatory responses and stimulating the proliferation of immune system cells, acting as immune modulators (Drayton et al. 2020). They are capable of recruiting and stimulating the proliferation of macrophages, neutrophils, eosinophils, activation of T lymphocytes and differentiation of dendritic cells, in addition to inducing or modulating pro-inflammatory cytokines and producing chemokines, chemotaxis, causing apoptosis and inhibiting the inflammatory response. This supports their use as potential therapeutic molecules against immune-related diseases (Liang and Diana 2020).

AMPs are thus able to act on several cell targets and are considered as promiscuous molecules in certain cases (Franco 2011). Multiple functions can be related to a single peptide structure, contrary to what was supposed years ago, which held that peptides have an unconditional structure directly associated with a particular function. Knowledge about AMPs promiscuity has been gaining ground in several fields of research, such as in antibiotic development (Silva et al. 2011; Franco 2011). Therefore, natural AMPs have enormous potential as an alternative approach for the development of new therapies, acting alone or in synergy with conventional drugs (de Oliveira et al. 2020; León-Buitimea et al. 2020).

Several studies have already demonstrated AMPs' therapeutic efficacy (Vilas Boas et al. 2017; Lima et al. 2017; Fensterseifer et al. 2019; León-Buitimea et al. 2020; Almeida et al. 2020). Another important feature is that AMPs are less susceptible to microbial resistance since they act on evolutionarily conserved cell membrane components. Therefore, bacterial cells would need different mutations over an extended period to completely redesign the structure of their cell membranes (Mercer et al. 2020; Mahlapuu et al. 2020). Thus, AMPs seem to have great potential

for antibiotic adjuvants, making it possible to reduce or circumvent the occurrence of antibiotic resistance. The synergy between AMPs and antibiotics, at lower dosages, can provide a reduction in the toxicity or adverse side effects of a drug (Browne et al. 2020).

Despite being less common, some anionic AMPs have been reported, such as the anionic AMP maximin-H5, isolated from amphibians, and dermcidin, secreted by the human eccrine sweat glands (Rios et al. 2016; Boparai and Sharma 2019). They are made up of negatively charged glutamic and aspartic acid residues, with a net negative charge ranging from −1 to −7. Precisely because of its negative charge, its mode of action differs from cationic AMPs. However, some anionic AMPs can be disruptive to bacterial cell membranes. These peptides are capable of using metal ions to form cationic salt bridges with the negative microbial membrane constituents, which enables cell penetration. When they reach the cytoplasm, they can bind to intracellular components such as ribosomes or inhibit ribonuclease activity, inducing cell death (Wang et al. 2019; Boparai and Sharma 2019; Moretta et al. 2021).

2.3 Classification

AMPs can be classified according to their structural aspects into three major subgroups: α-helical, β-sheet, and extended peptides (Tornesello et al. 2020; Moretta et al. 2021). The α-helical peptides, when in interaction with bacterial membranes, can be organized into a flexible amphipathic structure (Koo and Seo 2019; Cardoso et al. 2021). Examples of α-helical AMPs include magainin, temporins and melittin (Ge et al. 1999; Raja et al. 2017; Ramirez et al. 2019). Peptides in β-sheets may have greater structural stability in solution, due to the cysteine residues that are conserved and form disulfide bonds, which minimize proteinases degradation. This class includes AMPs such as protegrins from the cathelicidin family, defensins and tachyplesins (Kumar et al. 2018; Seyfi et al. 2020). Extended peptides are composed of a large proportion of proline and glycine residues and have no specific secondary structure, but when in contact with membranes, they generally form an amphipathic helical structure (Koo and Seo 2019). Proline-rich short-chain extended peptides, such as indolicidin and tritrpticin, can be isolated from mammals and from insects, such as apidaecin. Glycine-rich extended peptides can be isolated from insects and have sizes ranging from 8 to 30 kDa (Wang et al. 2019).

3 Upstream Process Development

Large-scale AMPs production is definitively a challenging task. The direct isolation and purification of these molecules from natural sources is normally extremely labor-intensive, in addition to resulting in low yields (Wibowo and Zhao 2019). Chemical synthesis, including Fmoc and other methodologies, is one of the main methods

currently used to obtain purified AMPs with high biological activity. Despite providing a high yield and level of purity, the high manufacturing cost of this technique is a limiting factor for the development of AMPs as biopharmaceuticals, particularly for peptides with more than 35 residues and that have post-translational modifications (Deng et al. 2017; Wibowo and Zhao 2019). Peptide synthesis by SPPS (Solid Phase Peptide Synthesis) is a complex and expensive production technique, despite being efficient. AMPs manufacture by SDDS is estimated to cost around US$50–400 per gram of amino acid produced (Moretta et al. 2020). Alternative approaches that increase production and make the AMPs development process cheaper are necessary (Sinha and Shukla 2019).

Upstream bioprocessing consists of several stages to achieve high yield and final product quality. Through recombinant DNA technology, scalable, economical, and sustainable AMPs production is possible. This strategy allows the cloning of foreign genes into specific vectors for expression in host systems, such as bacteria and yeast (Jozala et al. 2016; Wibowo and Zhao 2019; de Oliveira et al. 2020). The positive screening and selection of clones is performed, as well as small-scale assays to evaluate cell growth and protein product levels. Lastly, bioprocesses are conducted in bioreactor systems for large-scale production, and batch, fed-batch, and continuous strategies are applied for the mass production of the recombinant AMPs of biopharmaceutical interest (Gronemeyer et al. 2014; Jozala et al. 2016; Tripathi and Shrivastava 2019). Figure 1 exemplifies the upstream bioprocess of AMPs biopharmaceutical manufacturing.

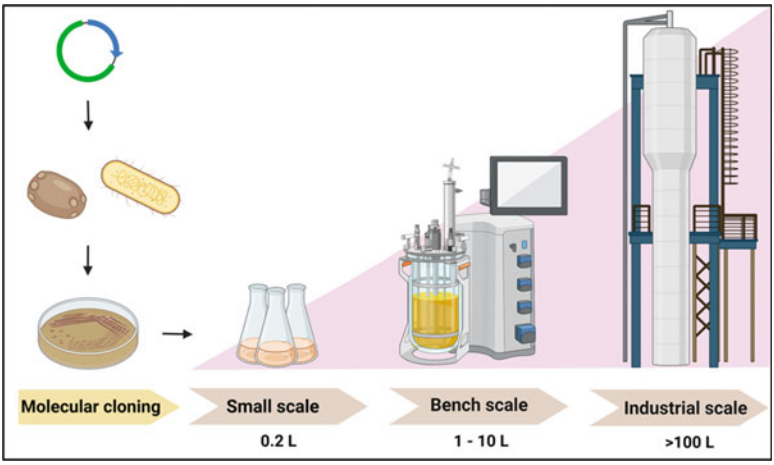

Fig. 1 Upstream bioprocess of AMPs biopharmaceutical manufacturing: molecular cloning, small-scale assays and scale-up of bioprocess. The figure was created by BioRender

3.1 Recombinant AMP Production in Microbial Cells

Bacterial expression systems are the type most used for heterologous gene expression since they are capable of producing a high level of recombinant molecules, show rapid multiplication, and have a simple media requirement (Gomes et al. 2016). Among them, the Gram-negative bacterium *E. coli* is the most extensively used for amplifying recombinant peptides' gene expression (Terpe 2006; Ahmad et al. 2018). In this regard, ~99% of proteins and peptides deposited in the Protein Data Bank (PDB) were produced in the *E. coli* expression system (de Oliveira et al. 2020). Under ideal culture conditions, bacterial cell number practically doubles every 20 min (Sezonov et al. 2007). Additionally, this host is also attractive due to its well-established genetic and expression protocols, wide availability of commercial expression vectors and cost-effectiveness (Sinha and Shukla 2019). There are several reports in the literature about the use of *E. coli* cells for recombinant AMPs expression. Recently, the proline-arginine-rich cationic peptide PR-39 was expressed fused to the SUMO or intein-chitin binding domain (CBD) in *E. coli* BL21 (DE3) pLysS, aiming to compare the expression level. Comparing the fusion protein concentration and single-step purification aspects, the intein system was better, even though similar amounts of pure PR-39 were recovered (250 μg L^{-1} of SUMO and 280 μg L^{-1} of intein) (Azari et al. 2020).

The *E. coli* (Rosetta DE3) system was also chosen to express more than 250 μg mL^{-1} of the human β-defensin 118 (DEFB118). This defensin is related to epididymal innate immunity, protecting the sperm against microorganism attacks in both male and female reproductive tracts. Antimicrobial assays demonstrated that DEFB118 activity occurred against Gram-negative and -positive bacteria at a minimum inhibitory concentration (MIC) of 4 μg mL^{-1} (Lin et al. 2020). Also using *E. coli* BL21 (DE3), a group of scientists created a modular system to express the lanthipeptide mersacidin, produced by *Bacillus amyloliquefaciens*. Mersacidin has bactericidal activity against several Gram-bacteria species, including methicillin-resistant *S. aureus* (Viel et al. 2021).

In recent years, *Bacillus subtilis* has been used as an alternative to the *E. coli* expression system for recombinant AMP production. *B. subtilis* is an endospore-forming Gram-negative soil bacterium and is generally recognized as safe (GRAS) because it lacks endotoxins, so it has been exploited as a production host for aquaculture industries (Pan et al. 2016; Cui et al. 2018). One of the greatest advantages of this bacterium is the possibility of naturally secreting the recombinant peptide (simplifying the downstream process), reducing hydrolysis of cell-associated proteins. Moreover, it simplifies the detection and purification processes of the target molecule (Zhang et al. 2020). Besides, *B. subtilis* does not produce lipopolysaccharides (LPS), preventing some degenerative disorders in humans and animals (Gomes et al. 2016).

Although bacterial cell-based expression systems are mostly used for the production of recombinant molecules, they are more prone to the degradation of cationic peptides (Li 2011). In addition, *E. coli* cells can produce inclusion bodies formed by

insoluble protein aggregates that hinder the recombinant AMP extraction and purification process (Gomes et al. 2016). Although engineered *E. coli* expresses periplasmic disulfide bond isomerase (DsbC) in the cytoplasm or through exporting the periplasm recombinant peptide (Abbas et al. 2013), this bacterium does not carry out other complex post-translational modifications (Parachin et al. 2012). Thus, for cases where there is a need for post-translational modifications, other systems should be chosen, such as yeasts or bacterial strains engineered for this purpose.

In addition to post-translational modifications, yeasts can carry out the correct protein folding. However, they have as a disadvantage the hyperglycosylation mechanisms and the need for aerobic fermentation, which reduces the growth rate, resulting in a lower recombinant protein yield (Juturu and Wu 2018). *S. cerevisiae* (baker's yeast) is a model organism for heterologous expression because its cell biology, genetics and biochemistry are well described (Gomes et al. 2016). This yeast is a suitable expression system for recombinant peptide production, as demonstrated by a group of scientists (Jiang et al. 2021). They expressed the cecropin P1, a positively charged α-helical peptide, isolated from the nematode *Ascaris suum*, which shows activity against *E. coli*, *Salmonella* sp., *Shigella* sp., and *Pasteurella* sp. In addition, this peptide also has antiviral activity against the PRRSV NADC30-like strain (Jiang et al. 2021). Defensins have also been successfully expressed in *S. cerevisiae*. By using the *MET17* promoter, Møller and colleagues expressed β-defensin-2 in *S. cerevisiae* cells (Møller et al. 2017).

In recent decades, the yeast *Pichia pastoris* (reclassified as *Komagataella phaffii)* (Naumov et al. 2018) has been extensively used for heterologous production of peptides and proteins, as it is capable of performing disulfide bridges, O- and N-glycosylation, and the correct processing of signal sequences (Wibowo and Zhao 2019). Several peptides have been expressed in a *K. phaffii* system (Wang et al. 2009; Basanta et al. 2010; Zhao et al. 2015; Zhang et al. 2018). Tachyplesin I (TP-I), a cationic peptide isolated from the Japanese horse crab (*Tachypleus tridentatus*) hemocytes, which inhibits the bacterial lipopolysaccharide (Li et al. 2019), was expressed in this system. Another example is the expression of the immunomodulatory and anti-inflammatory hybrid peptide (IAHP) LL-37Tα1. This peptide was efficiently produced in *K. phaffii* cells, demonstrating their ability to produce recombinant bioactive peptides (Ahmad et al. 2019).

Even though the choice of expression system must be made considering recombinant molecule properties, systems based on prokaryotic and eukaryotic cells have both strengths and weaknesses. The disadvantages can be overcome with multiple strategies, aimed at increasing recombinant molecule production. Codon optimization for specific organisms or engineered strains, the use of strong promoters and multimeric AMP expression in tandem or fused to a higher molecular mass protein are strategies that allow for a stable and high level of recombinant production (Fig. 2) (Deng et al. 2017). Below, we will address all these strategies to improve recombinant AMPs production.

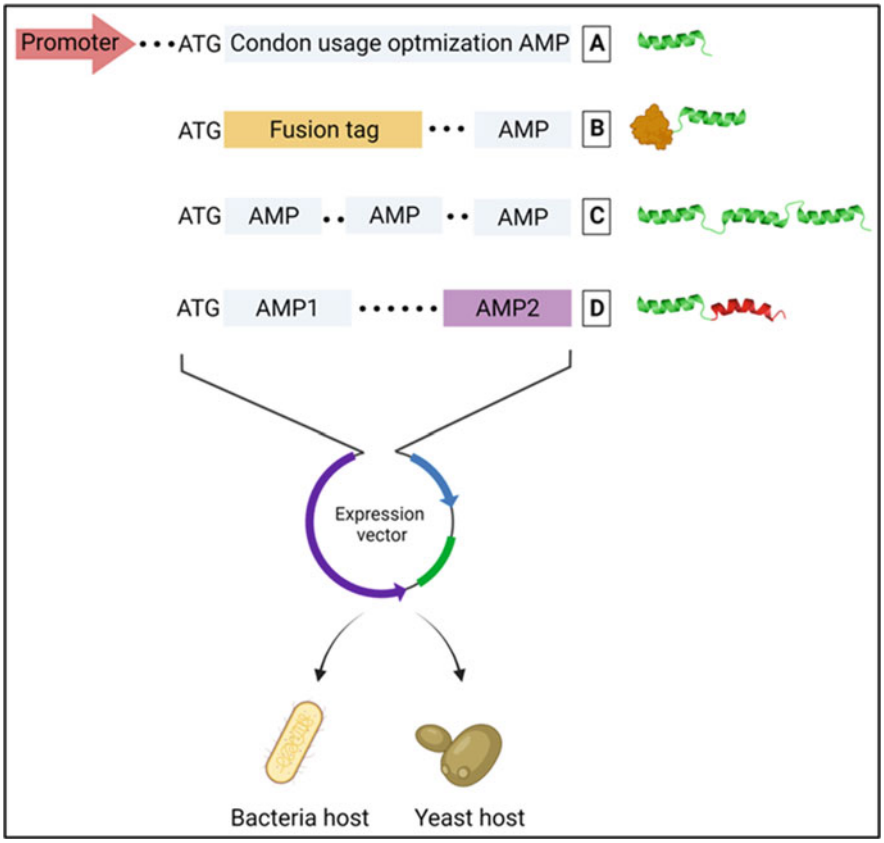

Fig. 2 Multiple strategies to increase the production of recombinant AMPs. (A) The use of strong promoters and codon optimization, (B) fusion to a higher molecular mass protein, (C) multimeric AMP expression in tandem and (D) hybridization of different AMPs. The figure was created by BioRender

3.2 Strategies to Enhance the Heterologous Expression Level

3.2.1 Cell Line Engineering and Host Strain

The choice of strain affects recombinant production success. At the end of the twentieth century, various potentially advantageous characteristics for heterologous production were tested in *E. coli* strains, generating line B, of which BL21(DE3) is the favorite host (Rosano et al. 2019). *E. coli* BL21 (DE3), and other parental B strains, have a deficiency in Lon protease, preventing exogenous protein degradation, an advantage to this system. In addition, the gene encoding the outer membrane protease OmpT is absent from the *E. coli* BL21 (DE3) genome, preventing the degradation of extracellular proteins (Rosano and Ceccarelli 2014). Another

advantage of the BL21 strain is that it expresses genes under the RNA polymerase promoters' control, such as lac, tac, trc, ParaBAD, PrhaBAD, T5, and T7 (de Oliveira et al. 2020). BL21(DE3) is able to provide a high AMP expression level, as observed in a study in which the yield of peptide P-113, derived from human saliva protein histatin 5, was 4 mg L^{-1} of bacterial suspension (Cheng et al. 2018).

Moreover, by transforming the *B. subtilis* (WB800) strain with a recombinant plasmid pHT-CI-CBF, scientists expressed cathelicidin-BF (CBF), a peptide isolated from snake venom (*Bungarus fasciatus*), fused with intein (Vogt et al. 2016). Data demonstrated that the intein expression system is a safe and efficient method by which to produce recombinant proteins in *B. subtilis,* since the yield of fusion CBF secreted reached ~0.5 mg L^{-1} (He et al. 2015). In addition to secretory capacity, this system can be exploited to produce recombinant molecules exposed on biofilm surface. Vogt and colleagues selected peptidic regions from tropomyosin and paramyosin, from the parasite *Echinococcus granulosus*, producing the peptides EgTrp and EgA31, respectively. They fused these peptides directly to the TsaA C-terminus, an important matrix protein (Vogt et al. 2016).

B. subtilis cells were genetically modified by the addition of a small tobacco etch virus (TEV) protease and the two-cistron expression vector gene of abaecin, previously isolated from *Apis mellifera* and further fused to TEV (Li et al. 2017). It has also been demonstrated that an expression system based on *B. subtilis* cells is capable of expressing a eukaryotic non-ribosomal peptide synthetase (esyn) gene that codes for the biosynthesis of the enniatin molecule (Zobel et al. 2015). Such examples reinforce the versatility of this system to produce recombinant peptides. In *K. phaffii*, recombinant peptides and proteins can be degraded through proteolysis mechanisms during transport or even in the extracellular space. To circumvent this limitation, protease-deficient strains such as SMD1163 (pep4 prb1 his4), SMD1165 (prb1 his4), and SMD1168 (his4 pep4) can be used. Although they have reduced proteolytic activity, due to gene silencing, these strains grow more slowly than the wild type, in addition to having low transformation efficiency and short cell viability (Daly and Hearn 2005; Ahmad et al. 2014). Even so, *K. phaffii* recombinant peptides' yield can be higher, as in the case of defensin VpDef, isolated from the mollusk *Venerupis philippinarum*, which was produced at a concentration of 60 μg mL^{-1} of culture medium (Meng et al. 2018). Another strategy to improve recombinant AMP yield is the use of both strong constitutive and inducible promoters.

3.2.2 Promoters and Codon Usage Optimization Strategies

The DNA region responsible for given gene transcription, or promoter, is another important component for recombinant AMPs production. Certain hosts have compatibility with different types of promoters. There is currently a plethora of commercially available promoters. Promoter T7, commonly found in pET series expression vectors, is a strong promoter most used for AMPs expression in *E. coli* cells (Deng et al. 2017). Promoters lac, tac, lacUV5, and T5 are also widely used

today, and research for the development of new and more efficient promoters continues to be developed. Inducible promoters were developed and derived from the constitutive phage promoters T5 (T5N25) and A1 (T7A1), capable of being recognized by RNAP of *E. coli* σ^{70}. Promoters not only decreased the basal expression but also increased the production of the recombinant protein (Schuller et al. 2020).

Another strategy to increase gene expression levels in bacterial systems is the use of dual promoters. Unlike eukaryotic systems, which have two separate expression cassettes, the dual promoters used in prokaryotic organisms are chimeric and are located, in tandem, upstream from the gene to be expressed (Öztürk et al. 2017). Using an optimized dual-promoter (P_{HpaII}–$P_{amyQ'}$) system, a group of scientists has demonstrated the increasing extracellular expression of β-CGTase, pullulanase, and α-CGTase in the *B. subtilis* (CCTCC M 2016536) strain. This strain was genetically manipulated to delete the *srfC*, *spoIIAC*, *nprE*, *aprE,* and *amyE* genes (Zhang et al. 2017).

In yeasts, the use of constitutive and inducible promoters is common. When compared to constitutive promoters, the inducible promoters are more used, because they allow a certain control over gene expression, which ends up resulting in a higher yield of recombinant peptides. Constitutive TEF1 and GPD promoters are the ones most used for *S. cerevisiae* expression. Although they can cause aggregation of folded proteins, making their secretion difficult, the promoters ADH1, GAPDH, PGK1, TPI, ENO, PYK1 also present an alternative (de Oliveira et al. 2020). Another factor that can influence the efficiency of promoters is polymorphism, which is already being evaluated (de Paiva et al. 2018).

Although the use of strong promoters could increase the heterologous expression levels, alternative strategies such as codon usage optimization can be used to boost the recombinant peptide expression. The codon usage bias refers to the availability of certain codons over others in the genome of organisms (Hanson and Coller 2018), and it can be a challenge for heterologous expression. Different species have a frequency of codons in a DNA sequence that is positively related to the corresponding tRNA, and the tRNA concentration in a cell is decisive for the number of amino acids accessible for protein translation extension (Fu et al. 2020).

The codon optimization approach had been used, in synthetic biology and the metabolic and cellular engineering fields, as an alternative by which to enhance heterologous gene expression levels (Lanza et al. 2014). In other words, codon optimization is suggested as a crucial factor in gene expression, since it consists of modifying synonymous codons through genetic engineering techniques, resulting in increased protein up-regulation and RNA levels (Mauro and Chappell 2014; Zhoua et al. 2016). In addition to contributing to mRNA stability, codon optimization also can impact ribosome translocation, connecting the processes of translation, elongation, and decay (Presnyak et al. 2015). Due to its importance to heterologous gene expression, scientists have developed, in recent years, new tools to facilitate codon optimization of synthetic genes. Such approaches explore the self-learning capacity of artificial intelligence (Tian et al. 2017; Fu et al. 2020), computational procedures (Chung and Lee 2012), and mathematical algorithms (Taneda and Asai 2020; Sen

et al. 2020). Organisms such as *B. subtilis* have another advantage, since the bias in codon usage is not a determining factor for heterologous expression. Additionally, transcription, translation, folding, and protein secretion processes, as well as methods of genetic manipulation and large-scale bioprocesses of this organism, are well described in the literature (Gomes et al. 2016).

Just changing a single codon synonym is enough to increase protein expression. The introduction of single codon synonym mutation (TCT → AGT) in the gene encoding the mrTNF-PADRE recombinant vaccine resulted in their enhanced production (~30% of total *E. coli* proteins) (Chu et al. 2018). It is also possible to improve translation efficiency in *S. cerevisiae* using the "condition-specific codon optimization" approach, as demonstrated by Lanza and colleagues (Lanza et al. 2014).

3.2.3 Tandem Multimeric Expression and Fusion Proteins

Another strategy commonly used for enhancing recombinant AMPs production is the tandem multimeric expression (Deng et al. 2017). Tandem peptide expression is an approach used for both prokaryotic and eukaryotic (yeasts) systems (Zhou et al. 2005; Fida et al. 2009; Wang et al. 2012). The expression of peptide LfcinB15-W4,10, a bovine lactoferricin, in four tandem repeats is more effective than monomers and the other repeats (2–8). At the end of the purification process, 10 mg of the tetramer with 99% purity was achieved (Tian et al. 2007). As demonstrated in the previous work, although it is possible to improve AMPs expression through multimeric tandem expression, the synthesis efficiency is not, unfortunately, proportional to copy number (Lee et al. 2002).

However, another strategy to overcome the low yield of recombinant AMPs is by expressing them fused to other proteins called fusion proteins which have been widely used in the expression of heterologous AMPs (Beaulieu et al. 2007; Liu et al. 2011; Mulder et al. 2015; Sousa et al. 2016; Xiao et al. 2017; Kaur et al. 2020). In addition to increasing the production level, these proteins facilitate the solubilization and purification of recombinant molecules (Costa et al. 2014). The small ubiquitin modifying (SUMO) fusion protein has an important function in AMPs' solubilization (Butt et al. 2005). Like SUMO, thioredoxin (Trx), a fusion protein from *E. coli*, also facilitates the solubilization of recombinant AMPs. However, unlike the former, Trx allows an increased expression rate (LaVallie et al. 1993; Costa et al. 2014). Another strategy is the use of polyhistidine ($_6$His) tag in the C- or N-terminal regions of a recombinant peptide. Although this tag is not a fusion protein, it facilitates the purification and detection by the western blot technique (Li 2011; Tavares et al. 2012; Belguesmia et al. 2020; Costa Ramos et al. 2021; Zhan et al. 2021).

3.2.4 AMP Hybridization

Hybridization, a combination of two native peptides, or even derivatives of hybrid peptides, is a method that has been used to produce new hybrid AMPs, in order to increase their antibacterial action, with reduced cytotoxicity (Wu et al. 2014; Klubthawee et al. 2020). A new hybrid AMP combined the α-helical fragments from peptides BMAP-27 and OP-145, producing the peptide H4, with the aim of maintaining its potent antimicrobial activity and reducing the cytotoxic profile against mammalian cells. The peptide H4 demonstrated activity against a broad spectrum of both Gram-negative and -positive bacteria, including the multidrug-resistant bacterial strains, in the range of 2.5–25 μM (Almaaytah et al. 2018).

Furthermore, the combination of two peptides can increase the plasma membrane permeabilization of the "parent" peptide. In order to evaluate the potential increase in activity of the hybrid peptides, hybrids were developed by joining membrane permeabilizing peptides (parasin or magainin 2) with membrane translocating peptides (DesHDAP1 or BF2). The results suggest that the permeabilizing activity is increased when the parent permeabilizing peptide is placed at the N-terminus, and through the addition of an alanine spacer between the sequences of the two parent peptides (Wade et al. 2019). Hybrid AMPs can also increase both the selectivity and stability of the molecule when compared to naturally occurring peptides (Yang et al. 2020). Through bioinformatic analysis, the 3.35 kDa hybrid magainin-thaumatin (MT) peptide was designed and expressed in *E. coli* BL21 (DE3) cells. Recombinant MT showed an inhibitory effect against *S. aureus*, *E. coli* DH5α, and *B. subtilis* at the MIC of 6.5, 20, and 9 μM, respectively (Tian et al. 2019).

Some hybrid peptides have strong antimicrobial activity against bacterial hosts, requiring the use of other expression systems. In this context, from hybridized plantaricin E (PlnE) and plantaricin (PlnF), type IIb bacteriocins, a ~5 kDa EF-1 hybrid peptide was developed (Li et al. 2020). This peptide can directly induce cell membrane permeabilization of *E. coli* cells. Expressing the recombinant EF-1 in the *K. phaffii* host, they recovered a yield of 32.65 mg L^{-1} with a purity of 94.9%. In addition to the bactericidal activity against enterohemorrhagic *E. coli* (EHEC) (MIC = 6.25 μM) and *E. coli* K88 (MIC = 3.125 μM) cells, recombinant EF-1 has no hemolytic activity (Li et al. 2020). The hybridization approach has the great potential to overcome some drawbacks, and it has been widely used (Jin et al. 2006; Xu et al. 2007; Arbulu et al. 2019; Agbale et al. 2019). Even though these strategies may have limitations, their use, alone or in combination, can increase the yield of recombinant AMPs, revolutionizing the production of biomolecules of medical and pharmaceutical interest.

4 Scale-Up from Small- to Large-Scale Fermentation

Bioprocess engineering has made considerable progress due to the high market demand for new biopharmaceuticals. New technologies related to bioprocessing techniques have been acquired for the large-scale production of proteins and peptides with biopharmaceutical potential for the treatment of various diseases. Economically viable production systems, which allow high yields to be achieved while maintaining the desired product quality, are now of great interest to bioprocesses industries (Potvin et al. 2012; Love et al. 2018; Tripathi and Shrivastava 2019).

An important step in the development process is the selection of a proper cell clone for the final production, which needs to fit the product quality requirements, processability and volumetric productivity. Clones can be selected for cell-specific and volumetric productivity, glycosylation profiles, aggregate formation, protein sequence heterogeneity and clone stability, among others. Cell culture conditions are decisive in productivity and product quality (Gronemeyer et al. 2014). AMPs biomanufacturing initially occurs on a small-scale, using shake flasks for expression system development. After this step, bioreactors are used to increase cell density and, consequently, the yield of the recombinant molecule of interest (Wibowo and Zhao 2019).

A defensin-like-peptide-P2 was successfully produced in *K. phaffii* using shake flasks. The induction of the recombinant peptide was performed with 0.5% methanol (v/v) every 24 h during the 120 h induction period, obtaining an expression level of 108.05 mg L^{-1}. Then, the process was scaled up, cultivation was carried out in a 5 L bioreactor, and a total of 1.69 g L^{-1} of peptide P2 was achieved. P2 exhibited bacterial reduction activity of 80–97% against multi-resistant *S. aureus* in RAW264.7 macrophages, among other activities (Yang et al. 2019).

Bacterial-based cell systems have also been used to express recombinant AMPs in shake flasks. One example is fowlicidin-2 expressed in *E. coli* BL21 (DE3). The induced expression of the peptide occurred by the addition of IPTG at a final concentration of 0.3 mM for 4 h in Luria-Bertani (LB) medium, at a temperature of 37 °C, under agitation. The results indicate a yield of 202 mg L^{-1} of the peptide of interest. The recombinant peptide demonstrated significant antimicrobial activity against a wide range of Gram-negative and -positive bacteria (Feng et al. 2015). Production of several other peptides was achieved on a small-scale using shake flasks (Sang et al. 2017; Meng et al. 2019).

Controlling certain parameters during production processes can make a big difference in AMPs yield. Monitoring critical operating parameters, including agitation, aeration, dissolved oxygen (DO), temperature, pH, and feed, is important and can be controlled in bioreactors. By monitoring the available oxygen rate, for example, it is possible to increase the availability of oxygen when it is low due to high cell density (Wibowo and Zhao 2019; Tripathi and Shrivastava 2019). The stability of these parameters allows high cell density and greater specific yield with a quality product to be achieved. In view of this, the physiological characterization of

production strains is essential for the proper development of a bioprocess (Gupta and Shukla 2017; Tripathi and Shrivastava 2019).

The composition of the culture medium also significantly influences cell growth and protein yield. The optimal selection of sources of carbon, nitrogen, salts, minerals and some growth factors and their proper concentrations are essential to achieve higher cell density and a higher level of recombinant proteins. The different carbon sources that are used as the main components of the cultivation medium in bioproduction, for instance, significantly affect cell metabolism, protein production and quality (García-Ortega et al. 2019). Complex, chemically defined or even semi-defined culture media can provide a nutrient-rich environment (Wibowo and Zhao 2019).

The parameters for obtaining a greater expression level of the hybrid magainin-thanatin (MT) recombinant AMP, produced in *E. coli*, were evaluated in shake flasks. Induction time (0, 1, 2, 3, 4, 5, and 6 h), temperature (32, 35, 37, 39 and 42 °C), IPTG concentration (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM) and the culture medium were evaluated separately in different cultures. Maximum production of the MT was observed by cultivation in TB medium at 37 °C and induction with 0.8 mM IPTG for 5 h (Tian et al. 2019). In another work, the recombinant AMP UBI18-35, derived from the natural human AMP ubiquicidin, was also expressed in *E. coli* Rosetta (DE3) pLysS. SDS-PAGE electropherogram processing by densitometry demonstrated that the ideal conditions for the best production of AMP UBI18-35 include expression at 28 °C for 4 h after induction with 0.5 mM IPTG (Ashcheulova et al. 2018).

After carrying out the cultivation in shake flasks, cultures on larger scales are desired to obtain a superior recombinant AMPs yield. In this context, different bioprocess techniques can be employed. One example is a simple batch mode of cultivation in which the supply of essential nutrients only occurs at the beginning of bioprocess. Otherwise, in a fed-batch mode, nutrients are added at specific rates throughout the process. Commercial production of some antibiotics, such as penicillin, occurs in fed-batch. In continuous cultivations, the provision of nutrients to microorganisms constantly occurs through the addition of a fresh culture medium, and part of the culture is removed to collect the product simultaneously (Li et al. 2014b; Tripathi and Shrivastava 2019).

4.1 Batch Processes

Simple discontinuous batches can be useful in initial bioprocessing applications, for studies of the physiology and kinetic parameters of the microorganism. During this phase, which lasts between 24 and 30 h, the addition of the substrate occurs only at the beginning of the cultivation, which extends until it reaches a certain concentration of cells, or until the initial substrate is completely consumed during the process. As there is no other substrate addition during the procedure, cell density and yield are limited. Another disadvantage is that during this phase the accumulation of

secondary metabolites can occur, which can be toxic to the host microorganism, and may cause a limitation in obtaining the product of interest (Ÿztürk et al. 2016; Mears et al. 2017; Blunt et al. 2018).

Large-scale production of the recombinant AMP plantaricin (PlnE) expressed in *E. coli* BL21 (DE3) was performed in a bioreactor with a volume of 12 L of LB. Cultivation was carried out in a simple batch mode at 30 and 25 °C. The expression induction was done with IPTG (0.5 mM) for 3 h when OD600 reached 0.4. According to data presented, the yield of purified plantaricin E was 140 and 180 mg at 30 and 25 °C, respectively, in 12 L of LB after 3 h of induction (Pal and Srivastava 2015). To improve the expression level of the ABP-dHC-cecropin A in *E. coli*, large-scale bioprocess was performed. The production was carried out in a culture medium with 10 g L^{-1} of glucose in a 5 L bioreactor, at a stirring rate of 300 rpm, temperature at 37 °C, dissolved oxygen at 50% air saturation by cascade agitation between 300 and 800 rpm and pH 7.0. After 4 h of cultivation, the recombinant AMP production was performed using 0.5 mM IPTG and maintained for another 3 h. According to the results, 47.3 g L^{-1} of biomass was achieved in the batch, which is 14× more than the biomass obtained in culture flasks with LB medium. The amount of soluble protein recovered was 8643 mg L^{-1} (Zhang et al. 2016).

4.2 Fed-Batch Processes

The discontinuous fed-batch process allows a high biomass concentration to be obtained, and the product of interest is due to the possibility of extending process time, as well as the gradual feeding of the selected substrate in precise quantities (García-Ortega et al. 2019). Generally, it starts with a low concentration of substrate and, when its complete consumption occurs, more substrate is added to keep the fermentation process going without exceeding the ideal level. Product collection only happens at the end of the process, allowing for good sterilization conditions for the procedure (Li 2011). This cultivation strategy is advantageous for many microorganisms such as *E. coli*, *S. cerevisiae*, *B. subtilis*, and *K. phaffii* (Philip et al. 2017). In fed-batches, a simple batch phase is performed before feeding the system, in which the consumption of available substrate and accumulation of biomass will occur (Looser et al. 2015). When the initial carbon source is finished, the batch type is changed to fed-batch, in which the production of the recombinant protein will take place. Usually, the substrates used are the carbon source, together with minerals and trace elements needed (Ÿztürk et al. 2016). The feeding methods commonly used are continuous or constant, exponential and pulsed (García-Ortega et al. 2019).

During continuous and exponential feeding techniques, substrate supply is carried out constantly after simple batch bioprocess. Continuous feeding is based on nutrient addition at a continuous rate throughout the entire process. Exponential feeding allows the specific growth rate maintenance at a pre-defined level. The substrate amount needed for cells to reach the target concentration can be calculated

beforehand, and substrates are constantly supplied throughout the process (Ÿztürk et al. 2016). Due to nutrient addition during the procedure, there is a volume increase inside the bioreactor, causing a dilution of cell concentration. Thus, feeding is exponential due to exponential cell growth (D'Anjou and Daugulis 2000). Feedback feeding happens by monitoring indirect variables, such as oxygen or carbon dioxide dissolved in the system, or by the concentration of the carbon source, such as methanol or ethanol (Ÿztürk et al. 2016). Therefore, feeding can occur through pulses, according to dissolved oxygen increase rate, which indicates a metabolism reduction due to substrate depletion (Zhao et al. 2008; Looser et al. 2015).

The peptide NZX, a plectasin derivative, was expressed in *K. phaffii* (X-33) in a 5 L bioreactor, aiming to boost NZX production. After cell growth on glucose, the induction of NZW expression was performed in a fed-batch with methanol for 120 h. According to the results, the total biomass concentration obtained was 268 g $L^{-1,}$ and secreted protein was 2820 mg L^{-1}. The recombinant peptide showed high stability and low cytotoxicity, in addition to antimicrobial activity against *Staphylococcus hyicus,* both in in vitro and in in vivo experiments (Liu et al. 2020). Another group of scientists also used the yeast *K. phaffii* as a host for AMPs production. According to the data, the AMP clavanin MO was successfully expressed in *K. phaffii*. Therefore, the cultivation of the recombinant strain was then carried out in a bioreactor using BMGY medium with 40 g L^{-1} of glycerol in the discontinuous batch phase. After the total consumption of glycerol, the fed-batch carried out with 0.5% methanol added every 12 h during 72 h was initiated. At the end of the bioprocess, 5 mg mL^{-1} of the recombinant clavanin MO was recovered (Mulder et al. 2015).

Piscidin 4 (TP4), derived from tilapia *Oreochromis niloticus*, was produced in a 500 L bioreactor with a culture medium of basal salts with trace elements. In the fed-batch method, recombinant yeast *K. phaffii* growth was stimulated by adding glycerol as a carbon source until it ran out. Then, the discontinuous phase, also fed with glycerol, was conducted to obtain a higher cell density. Finally, the induction phase with 100% methanol was carried out for 65 h to induce the production of the recombinant TP4. Fed-batch bioprocess in recombinant strain *K. phaffii* (KM71) XS10 was also performed for the production of the recombinant bovine lactoferrampin–lactoferricin (LFA–LFC). The batch was kept for 12 h in a 10 L bioreactor with basal salts medium containing trace elements. After this step, a glycerol feed (50% (v/w)) was started at 10 mL/h for 12 h. Six hours later, peptide induction was initiated by the addition of 0.5% methanol (v/v) every 24 h. According to the results, it was possible to obtain about 1.025 $\pm$ 169 mg L^{-1} of LFA-LFC in the culture supernatant and about 53 $\pm$ 4 mg L^{-1} of the purified LFA-LFC (Tang et al. 2012).

4.3 Continuous Processes

Although relevant studies have already been published about discontinuous fed-batches, they can be less robust, more laborious and time-consuming. While the continuous mode allows for stable culture conditions, it provides a similar physiological state for all cells in the culture medium and, for these reasons, it has been one of the most widely used strategies for obtaining physiological data (García-Ortega et al. 2019). In the continuous mode, the target compound can be produced in large quantities due to the system maintenance at stationary phase. Fresh culture medium is added to the bioreactor, and part of the culture is continuously removed at a constant value: the dilution rate (D). This bioprocess is also known as chemostat (chemical and static environment), since the conditions inside the reactor (substrate, cell and product concentrations) can be stable (Mears et al. 2017; Blunt et al. 2018). Other parameters such as pH value, oxygen rate, working volume and nutrient supply are also kept constant (Koller 2018).

Continuous processes can keep the growth rate moderate and constant for long periods, which avoids the non-productive time spent on harvesting, cleaning, preparing a new medium, sterilizing and cooling the culture in a discontinuous process, generating higher average yield (Blunt et al. 2018). Among other advantages, it allows knowledge about bioprocess physiology, making it less laborious for the operation and maintenance of the process when it reaches a steady state (Blunt et al. 2018). Furthermore, the inhibition risk by substrate, or by-products, is lower as the final product is continuously collected throughout the process (Li et al. 2014a).

The interest in continuous cultures is not restricted to studies of microbial physiology and process development. It is also of great interest for the manufacture of recombinant proteins, as it allows cells to be kept in production states for a longer time, and consequently, the yield of the process significantly increases, while costs fall (Peebo and Neubauer 2018; Khanal and Lenhoff 2021). Several examples of continuous processes to produce recombinant proteins in different microorganisms have been reported in the scientific literature. However, some shortcomings associated with continuous processes make their commercialization limited (Rathore et al. 2015; Peebo and Neubauer 2018).

Both the stability and sterility during a long period of cultivation, as well as the lack of flexibility in the short term (caused by the need for long periods of execution), associated with the genetic inability of the cells can be cited as some limitations of this method of bioprocess (Rathore et al. 2015; Peebo and Neubauer 2018). Mainly due to these issues, continuous production of recombinant proteins is used particularly for the manufacturing of high-demand biopharmaceuticals. Recombinant insulin, produced in *S. cerevisiae* in the 1990s, is the only known example of a continuous industrial recombination process using microorganisms (Diers et al. 1991).

5 Purification of AMPs: Downstream Process Development

The downstream process consists of the recovery and purification of recombinant peptides, aiming to reduce costs (Clarke 2013). In this regard, innovative approaches have been applied in purification techniques of the downstream pharmaceutical industry (Gupta and Shukla 2017). These techniques are divided into three different stages: (a) an initial recovery (extraction or isolation), (b) purification (removal of most contaminants), and (c) polishing for removal of specified contaminants (Zydney 2016; Gupta and Shukla 2017; Tripathi and Shrivastava 2019). Taken together, all these steps improved the search, production and application of therapeutic peptides (Agyei et al. 2017). The purification process for recombinant AMPs is demonstrated in Fig. 3.

5.1 Recovery

At the recovery stage, the most common techniques applied in the industries are centrifugation, tangential flow microfiltration (MF-TFF), or depth filtration. The objective is to remove cells, fine particulates, colloids and soluble impurities prior to the initial purification steps (Pieracci et al. 2018). Generally, organisms such as yeasts export the recombinant peptides to the extracellular space, while in bacterial systems, the heterologous molecules are sent to the periplasmic space. To recover the extracellular recombinant peptide, it is necessary to concentrate it by a centrifugation

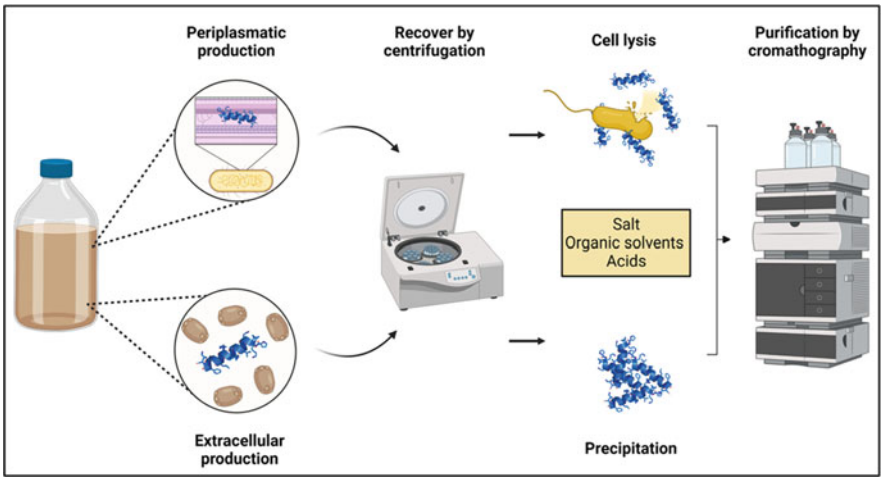

Fig. 3 Purification process of recombinant AMPs. The process addressed to both the periplasmic and extracellular spaces, through a signal peptide, can initially be recovered by centrifugation and, after this, from the cell lysis process (periplasm) or by precipitation (extracellular). The final step consists of the purification by chromatographic techniques. The figure was created by BioRender

or ultracentrifugation process. In addition to chromatography, the precipitation of the samples can be done to improve their concentration (Jozala et al. 2016).

The presence of a signal peptide located in the C or N-terminus region allows the export of the recombinant peptide to the extracellular (in *K. phaffii*) or periplasmic (in *E. coli*) space (Weinacker et al. 2013). For purified peptides in periplasmic space, the cells are submitted to lysis (sonication, high-pressure homogenizer, passing through mills, etc.) and clarification can be used to remove the cell debris. Therefore, the clarified product is purified using precipitation and/or chromatography. In some cases, recombinant peptides produced in periplasmic space can be accumulated as inclusion bodies (IBs), which are inactive, making an extra step necessary to refold the peptides to their native conformation (Ehgartner et al. 2017; Tripathi and Shrivastava 2019).

5.2 Purification

After the recombinant peptide recovery, the next step is the purification process by chromatography techniques (Tripathi and Shrivastava 2019). There are several types of chromatography, such as size exclusion chromatography, ion-exchange chromatography, low-pressure hydrophobic interaction chromatography, countercurrent distribution, partition chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC) (Tripathi and Shrivastava 2019). Although the chromatography techniques are well-established processes, they are more expensive, when compared to other purification approaches, as well as presenting limitations in throughput and scalability (Tripathi and Shrivastava 2019). To overcome some of those limitations, it is important that the purification strategy must be evaluated for every single case, and it can change according to the particular application (Carta and Jungbauer 2010). In this context, several AMPs have already been purified using chromatography techniques.

The host defense peptides (HDPs) IDR1, MX226, LL37, CRAMP, HHC-10, E5, and E6 were purified in a simplified 2-step purification method. After recombinant production in high yields, using *E. coli* strain BL21 with SUMO fusion, the peptides were first purified using a Ni-NTA Sepharose column. The recombinant peptide IDR-1 was successfully purified. From a yield of about 1.5 kg of wet biomass, 6% of the total protein produced is equivalent to the SUMO-IDR-1, with an estimated yield of 0.48 g/L of fermentation. Subsequently, the SUMO-specific protease sumoase was used to release the peptide from the fusion. Following the cleavage, reversed-phase chromatography was used to separate SUMO and sumoase from the peptide and recover the homogenously pure peptide (Bommarius et al. 2010).

6 Optimization of the Industrial Processes

Recently, different methods have been used in order to optimize the industrial process, such as high-throughput devices (HTD), design of experiments (DoE), and process analytical technology (PAT). The HTD method is divided into screening (HTS), process development and experimentation (HTPD), and experimentation (HTE). These processes together seek to obtain data quickly and reliably, doing tests on a small-scale, producing multiple data points to be generated with low usage of laboratory space, in addition to saving time (Silva et al. 2021).

HTS is responsible for producing hundreds of thousands of data points per day, using microtiter plates with 384 and 1536 wells (Mayr and Bojanic 2009). Another option is microfluidics, which use very small amounts of liquids, with quick analysis generating multiple data points and thus reducing time and laboratory space (Whitesides 2006). The goal of HTS is optimization of the process and delivering the final product actively and quickly (Shukla et al. 2017; Silva et al. 2021). HTPD makes use of complex computational tools to seek to establish a better design and understanding of the process (Bhambure et al. 2011). HTE is the union of HTS and HTPD to provide an overview of the miniaturization and automation process, enabling applicability in the biopharmaceutical industry (Silva et al. 2021; São Pedro et al. 2021).

In this regard, a group of scientists reported the combination of two different methods (high-throughput (HTP) processes and flow-mediated synthesis). They rapidly obtained a large amount of information about new peptides. They tested a library of potential AMPs against *P. aeruginosa*, and the results allowed them to identify the peptide with the highest antimicrobial activity. The workflow contributes to the discovery and optimization of peptide structures as new antimicrobial agents for biomedical applications (Judzewitsch et al. 2020). The suitability of both the diffusion self-interaction parameters (kDa) and osmotic second virial coefficients (B22) was investigated by using high-throughput screening, aiming to seek the peptides' formulation aggregation risk. In this study, thermal stress effects on colloidal stability were evaluated, as well as six buffering systems at two-selected pH values, four tonicity agents and a common preservative. Acetate and succinate buffer at pH 4.5, combined with glycerol or mannitol, demonstrated greater stability of peptides. Data suggest that HT methods display important information about the optimization of colloidal stability during the early development of peptide-based liquid formulations (Dauer et al. 2021).

In addition to the HTS method, DoE also allows bioprocesses to be optimized through monitoring and control of the experiment. DoE enables simultaneous evaluation of a large number of variables. Thus, this methodology obtains maximum information about the process, saving time and financial resources to obtain a high-quality product (Kasemiire et al. 2021). This methodology was used to optimize the AMP human β-defensin 2 (HBD2) expression yields in *E. coli* BL21 (DE3). The authors used 24-factorial design of experiments (DoE) to evaluate the following variables, namely cell density, temperature, induction period, and inducer

concentration. They got 19 different combinations, and the best condition was pre-induction temperature of 37 °C, a cell density of 1.0 U (600 nm), an induction temperature of 20 °C and a 0.1 mM of gene expression inducer (IPTG) over 4 h. Researchers concluded that these conditions produced the HBD2 peptide in a higher proportion than previously tested (Corrales-García et al. 2020).

Process Analytical Technology (PAT), initially from the US Food and Drug Administration (FDA), is responsible for the regulation of biopharmaceutical production, dealing with measuring, analyzing, monitoring, and ultimately controlling all substantial characteristics of a bioprocess (Scott and Wilcock 2006; Kornecki and Strube 2018). It is important to remember that the transition from up- to downstream in a bioprocess is always challenging. Any small change can affect final product quality. PAT was used for monitoring and controlling specific reactions during the manufacturing process of the recombinant lethal toxin-neutralizing factor (rLTNF), which acts against rattlesnake venom. Monitoring was performed in three critical production stages (solubilization of the IBs, enzymatic cleavage with α-chymotrypsin and quenching of the reaction at the optimal time). The authors reported that the process with PAT tools through various batches of rLTNF production would allow real-time analysis of quality and production control (Hebbi et al. 2020). Therefore, the methods described above are of great importance, as they seek to find the best operating conditions, in addition to determining the operating windows. These features are extremely useful in minimizing the impact of batch-to-batch variations and human error during the bioprocess.

7 Conclusions and Future Directions

Bioprocessing technology is a great opportunity for developing recombinant therapeutic AMPs on a large-scale. The promiscuity of these molecules makes them promising alternatives in the development of new biopharmaceuticals against a number of infectious diseases. The use of microorganisms and different fermentation processes allows large-scale high-quality AMPs production. Therefore, optimization of process conditions seems to be extremely important to generate biologically active and stable molecules. Different strategies can be applied for cell line engineering, such as promoters and codon usage optimization, tandem multimeric expression and fusion protein production, and AMP hybridization.

General optimization of AMPs bioprocessing seeks the best expression system and conditions for production and recovery of compounds. However, it must be emphasized that there is no ideal production system and that they all have advantages and disadvantages. Analysis of AMPs physicochemical characteristics is essential to find the most useful conditions to produce stable and active molecules, as well as in large quantity. The same occurs during the recombinant strain cultivation on a small or large-scale. Cultivation conditions and bioprocess strategies must be carefully analyzed, as they directly influence the recombinant AMPs expression.

The choice of an appropriate recovery method for an AMP after its production is also very important, as it can allow AMPs with a high degree of purity to be obtained.

In summary, optimization methods for industrial process are important developments for this field. Through best operating conditions, these optimized methods increase the production rate and reduce costs in both upstream and downstream process development. Such improvements are important because they can allow us to produce AMPs in high quantities and purity, and these will then be applied in the pharmaceutical industry, research, and pre-clinical and clinical trials. Furthermore, bioprocessing automation can reduce the impact related to variations during bioprocesses, as well as human error. All approaches related to upstream and downstream processes in the recombinant production of AMPs mentioned here generate lower production costs, manufacturing diversification and recombinant AMPs quality.

Acknowledgments This work was supported by the Coordination of Improvement of Higher Education Personnel (CAPES); National Council of Technological and Scientific Development (CNPq), Federal District Research Support Foundation (FAPDF) and Support Foundation for the Development of Education, Science and Technology of the State of Mato Grosso do Sul (FUNDECT).

References

Abbas A, Plattner S, Shah KH, Bohlmann H (2013) Comparison of periplasmic and intracellular expression of Arabidopsis thionin proproteins in *E. coli*. Biotechnol Lett 35:1085–1091

Agbale CM, Sarfo JK, Galyuon IK et al (2019) Antimicrobial and antibiofilm activities of helical antimicrobial peptide sequences incorporating metal-binding motifs. Biochemistry 58:3802–3812

Agyei D, Ahmed I, Akram Z et al (2017) Protein and peptide biopharmaceuticals: an overview. Protein Pept Lett 24:94–101

Ahmad M, Hirz M, Pichler H, Schwab H (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol 98:5301–5317

Ahmad I, Nawaz N, Darwesh NM et al (2018) Overcoming challenges for amplified expression of recombinant proteins using *Escherichia coli*. Protein Expr Purif 144:12–18

Ahmad B, Hanif Q, Xubiao W et al (2019) Expression and purification of hybrid ll-37tα1 peptide in *Pichia pastoris* and evaluation of its immunomodulatory and anti-inflammatory activities by LPS neutralization. Front Immunol 10:1–12

Almaaytah A, Qaoud MT, Abualhaijaa A et al (2018) Hybridization and antibiotic synergism as a tool for reducing the cytotoxicity of antimicrobial peptides. Infect Drug Resist 11:835–847

Almeida LHO, de Oliveira CFR, Rodrigues MS et al (2020) Adepamycin: design, synthesis and biological properties of a new peptide with antimicrobial properties. Arch Biochem Biophys 691:108487

Andersson DI, Hughes D, Kubicek-Sutherland JZ (2016) Mechanisms and consequences of bacterial resistance to antimicrobial peptides. Drug Resist Updat 26:43–57

Arbulu S, Jiménez JJ, Gútiez L et al (2019) Cloning and expression of synthetic genes encoding native, hybrid- and bacteriocin-derived chimeras from mature class IIa bacteriocins, by *Pichia pastoris* (syn. *Komagataella* spp.). Food Res Int 121:888–899

Arora S, Saxena V, Ayyar BV (2017) Affinity chromatography: a versatile technique for antibody purification. Methods 116:84–94

Ashcheulova DO, Efimova LV, Lushchyk AY et al (2018) Production of the recombinant antimicrobial peptide UBI 18-35 in *Escherichia coli*. Protein Expr Purif 143:38–44

Assis LM, Nedeljković M, Dessen A (2017) New strategies for targeting and treatment of multidrug resistant *Staphylococcus aureus*. Drug Resist Updat 31:1–14

Azari M, Asad S, Mehrnia MR (2020) Heterologous production of porcine derived antimicrobial peptide PR-39 in *Escherichia coli* using SUMO and intein fusion systems. Protein Expr Purif 169:105568

Basanta A, Gómez-Sala B, Sánchez J et al (2010) Use of the yeast *Pichia pastoris* as an expression host for secretion of enterocin L50, a leaderless two-peptide (L50A and L50B) bacteriocin from *Enterococcus faecium* L50▽. Appl Environ Microbiol 76:3314–3324

Beaulieu L, Tolkatchev D, Jetté JF et al (2007) Production of active pediocin PA-1 in *Escherichia coli* using a thioredoxin gene fusion expression approach: cloning, expression, purification, and characterization. Can J Microbiol 53:1246–1258

Belguesmia Y, Bendjeddou K, Kempf I et al (2020) Heterologous biosynthesis of five new class II bacteriocins from *Lactobacillus paracasei* CNCM I-5369 with antagonistic activity against pathogenic *Escherichia coli* strains. Front Microbiol 11:1–9

Bhambure R, Kumar K, Rathore AS (2011) High-throughput process development for biopharmaceutical drug substances. Trends Biotechnol 29:127–135. https://doi.org/10.1016/j.tibtech.2010.12.001

Bhopale GM (2020) Antimicrobial peptides: a promising avenue for human healthcare. Curr Pharm Biotechnol 21:90–96

Blunt W, Levin DB, Cicek N (2018) Bioreactor operating strategies for improved polyhydroxyalkanoate (PHA) productivity. Polymers (Basel) 10:1197

Bommarius B, Jenssen H, Elliott M et al (2010) Cost-effective expression and purification of antimicrobial and host defense peptides in *Escherichia coli*. Peptides 31:1957–1965

Boparai JK, Sharma PK (2019) Mini review on antimicrobial peptides, sources, mechanism and recent applications. Protein Pept Lett 27:4–16

Boto A, Pérez de la Lastra J, González C (2018) The road from host-defense peptides to a new generation of antimicrobial drugs. Molecules 23:311

Briand L, Marcion G, Kriznik A et al (2016) A self-inducible heterologous protein expression system in *Escherichia coli*. Sci Rep 6:33037

Browne K, Chakraborty S, Chen R et al (2020) A new era of antibiotics: the clinical potential of antimicrobial peptides. Int J Mol Sci 21:7047

Butt TR, Edavettal SC, Hall JP, Mattern MR (2005) SUMO fusion technology for difficult-to-express proteins. Protein Expr Purif 43:1–9

Cardoso P, Glossop H, Meikle TG et al (2021) Molecular engineering of antimicrobial peptides: microbial targets, peptide motifs and translation opportunities. Biophys Rev 13:35–69

Carta G, Jungbauer A (2010) Protein chromatography. Wiley, Chichester

Chen CH, Lu TK (2020) Development and challenges of antimicrobial peptides for therapeutic applications. Antibiotics 9:24

Cheng KT, Wu CL, Yip BS et al (2018) High level expression and purification of the clinically active antimicrobial peptide P-113 in *Escherichia coli*. Molecules 23:800

Chu C, Zhang W, Li J et al (2018) A single codon optimization enhances recombinant human TNF-α vaccine expression in *Escherichia coli*. Biomed Res Int 2018:1–8

Chung BKS, Lee DY (2012) Computational codon optimization of synthetic gene for protein expression. BMC Syst Biol 6:134

Clarke KG (2013) Downstream processing. In: Bioprocess engineering. Elsevier, Amsterdam, pp 209–234

Corrales-García LL, Serrano-Carreón L, Corzo G (2020) Improving the heterologous expression of human β-defensin 2 (HBD2) using an experimental design. Protein Expr Purif 167:105539

Costa Ramos LF, Rangel J, Andrade G et al (2021) Identification and recombinant expression of an antimicrobial peptide (cecropin B-like) from soybean pest *Anticarsia gemmatalis*. J Venom Anim Toxins Incl Trop Dis 27:1–12

Costa S, Almeida A, Castro A, Domingues L (2014) Fusion tags for protein solubility, purification and immunogenicity in *Escherichia coli*: the novel Fh8 system. Front Microbiol 5:1–20

Cui W, Han L, Suo F et al (2018) Exploitation of *Bacillus subtilis* as a robust workhorse for production of heterologous proteins and beyond. World J Microbiol Biotechnol 34:145

D'Anjou MC, Daugulis AJ (2000) Mixed-feed exponential feeding for fed-batch culture of recombinant methylotrophic yeast. Biotechnol Lett 22:341–346

da Costa JP, Cova M, Ferreira R, Vitorino R (2015) Antimicrobial peptides: an alternative for innovative medicines? Appl Microbiol Biotechnol 99:2023–2040

Daly R, Hearn MTW (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J Mol Recognit 18:119–138

Dauer K, Pfeiffer-Marek S, Kamm W, Wagner KG (2021) Microwell plate-based dynamic light scattering as a high-throughput characterization tool in biopharmaceutical development. Pharmaceutics 13:172

de Oliveira KBS, Leite ML, Rodrigues GR et al (2020) Strategies for recombinant production of antimicrobial peptides with pharmacological potential. Expert Rev Clin Pharmacol 13:367–390

de Paiva DP, Rocha TB, Rubini MR et al (2018) A study on the use of strain-specific and homologous promoters for heterologous expression in industrial *Saccharomyces cerevisiae* strains. AMB Express 8:82

Deng T, Ge H, He H et al (2017) The heterologous expression strategies of antimicrobial peptides in microbial systems. Protein Expr Purif 140:52–59

Diers IV, Rasmussen E, Larsen PH, Kjaersig I (1991) Yeast fermentation processes for insulin production. Bioprocess Technol 13:166–176

Dijksteel GS, Ulrich MMW, Middelkoop E, Boekema BKHL (2021) Review: lessons learned from clinical trials using antimicrobial peptides (AMPs). Front Microbiol 12:616979

Drayton M, Kizhakkedathu JN, Straus SK (2020) Towards robust delivery of antimicrobial peptides to combat bacterial resistance. Molecules 25:3048

Ehgartner D, Sagmeister P, Langemann T et al (2017) A novel method to recover inclusion body protein from recombinant *E. coli* fed-batch processes based on phage ΦX174-derived lysis protein E. Appl Microbiol Biotechnol 101:5603–5614

Feng X, Xu W, Qu P et al (2015) High-yield recombinant expression of the chicken antimicrobial peptide fowlicidin-2 in *Escherichia coli*. Biotechnol Prog 31:369–374

Fensterseifer ICM, Felício MR, Alves ESF et al (2019) Selective antibacterial activity of the cationic peptide PaDBS1R6 against Gram-negative bacteria. Biochim Biophys Acta Biomembr 1861:1375–1387

Fida HM, Kumada Y, Terashima M et al (2009) Tandem multimer expression of angiotensin I-converting enzyme inhibitory peptide in *Escherichia coli*. Biotechnol J 4:1345–1356

Franco OL (2011) Peptide promiscuity: an evolutionary concept for plant defense. FEBS Lett 585: 995–1000

Fu H, Liang Y, Zhong X et al (2020) Codon optimization with deep learning to enhance protein expression. Sci Rep 10:17617

García-Ortega X, Cámara E, Ferrer P et al (2019) Rational development of bioprocess engineering strategies for recombinant protein production in *Pichia pastoris* (*Komagataella phaffii*) using the methanol-free GAP promoter. Where do we stand? New Biotechnol 53:24–34

Ge Y, MacDonald DL, Holroyd KJ et al (1999) In vitro antibacterial properties of pexiganan, an analog of magainin. Antimicrob Agents Chemother 43:782–788

Gomes AR, Byregowda SM, Veeregowda BM, Balamurugan V (2016) An overview of heterologous expression host systems for the production of recombinant proteins. Adv Anim Vet Sci 4: 346–356

Gronemeyer P, Ditz R, Strube J (2014) Trends in upstream and downstream process development for antibody manufacturing. Bioengineering 1:188–212

Gupta SK, Shukla P (2017) Sophisticated cloning, fermentation, and purification technologies for an enhanced therapeutic protein production: a review. Front Pharmacol 8:1–17
Haney EF, Mansour SC, Hancock REW (2017) Antimicrobial peptides. Springer, New York
Hanson G, Coller J (2018) Codon optimality, bias and usage in translation and mRNA decay. Nat Rev Mol Cell Biol 19:20–30
He Q, Fu A, Li T (2015) Expression and one-step purification of the antimicrobial peptide cathelicidin-BF using the intein system in *Bacillus subtilis*. J Ind Microbiol Biotechnol 42: 647–653
Hebbi V, Kumar D, Rathore AS (2020) Process analytical technology implementation for peptide manufacturing: cleavage reaction of recombinant lethal toxin neutralizing factor concatemer as a case study. Anal Chem 92:5676–5681
Jiang R, Zhang P, Wu X et al (2021) Expression of antimicrobial peptide Cecropin P1 in *Saccharomyces cerevisiae* and its antibacterial and antiviral activity in vitro. Electron J Biotechnol 50:16–22
Jin F, Xu X, Wang L et al (2006) Expression of recombinant hybrid peptide cecropinA(1–8)–magainin2(1–12) in *Pichia pastoris*: purification and characterization. Protein Expr Purif 50: 147–156
Jozala AF, Geraldes DC, Tundisi LL et al (2016) Biopharmaceuticals from microorganisms: from production to purification. Braz J Microbiol 47:51–63
Judzewitsch PR, Corrigan N, Trujillo F, et al (2020) High-throughput process for the discovery of antimicrobial polymers and their upscaled production via flow polymerization. Macromolecules 53:631–639. https://doi.org/10.1021/acs.macromol.9b02207
Juturu V, Wu JC (2018) Heterologous protein expression in *Pichia pastoris*: latest research progress and applications. Chembiochem 19:7–21
Kasemiire A, Avohou HT, De Bleye C et al (2021) Design of experiments and design space approaches in the pharmaceutical bioprocess optimization. Eur J Pharm Biopharm 166:144–154
Kaur J, Kumar A, Kaur J (2018) Strategies for optimization of heterologous protein expression in *E. coli*: roadblocks and reinforcements. Int J Biol Macromol 106:803–822
Kaur N, Dilawari R, Kaur A et al (2020) Recombinant expression, purification and PEGylation of Paneth cell peptide (cryptdin-2) with value added attributes against *Staphylococcus aureus*. Sci Rep 10:12164
Khanal O, Lenhoff AM (2021) Developments and opportunities in continuous biopharmaceutical manufacturing. MAbs 13:1903664
Khow O, Suntrarachun S (2012) Strategies for production of active eukaryotic proteins in bacterial expression system. Asian Pac J Trop Biomed 2:159–162
Kimple ME, Brill AL, Pasker RL (2013) Overview of affinity tags for protein purification. Curr Protoc Protein Sci 73:9.9.1–9.9.23
Klubthawee N, Adisakwattana P, Hanpithakpong W et al (2020) A novel, rationally designed, hybrid antimicrobial peptide, inspired by cathelicidin and aurein, exhibits membrane-active mechanisms against *Pseudomonas aeruginosa*. Sci Rep 10:9117
Koller M (2018) A review on established and emerging fermentation schemes for microbial production of polyhydroxyalkanoate (PHA) biopolyesters. Fermentation 4:1–30
Koo HB, Seo J (2019) Antimicrobial peptides under clinical investigation. Pept Sci 111:1–15
Kornecki M, Strube J (2018) Process analytical technology for advanced process control in biologics manufacturing with the aid of macroscopic kinetic modeling. Bioengineering 5:25
Kumar P, Kizhakkedathu J, Straus S (2018) Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. Biomol Ther 8:4
Lanza AM, Curran KA, Rey LG, Alper HS (2014) A condition-specific codon optimization approach for improved heterologous gene expression in *Saccharomyces cerevisiae*. BMC Syst Biol 8:33
LaVallie ER, DiBlasio EA, Kovacic S et al (1993) A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. Bio/Technology 11:187–193

Laws M, Shaaban A, Rahman KM (2019) Antibiotic resistance breakers: current approaches and future directions. FEMS Microbiol Rev 43:490–516

Lee J, Kim M, Cho J, Kim S (2002) Enhanced expression of tandem multimers of the antimicrobial peptide buforin II in *Escherichia coli* by the DEAD-box protein and trxB mutant. Appl Microbiol Biotechnol 58:790–796

Lee EY, Lee MW, Fulan BM et al (2017) What can machine learning do for antimicrobial peptides, and what can antimicrobial peptides do for machine learning? Interface Focus 7:20160153

Leite ML, Sampaio KB, Costa FF et al (2019) Molecular farming of antimicrobial peptides: available platforms and strategies for improving protein biosynthesis using modified virus vectors. An Acad Bras Cienc 91:1–23

León-Buitimea A, Garza-Cárdenas CR, Garza-Cervantes JA et al (2020) The demand for new antibiotics: antimicrobial peptides, nanoparticles, and combinatorial therapies as future strategies in antibacterial agent design. Front Microbiol 11:1–10

Li Y (2011) Recombinant production of antimicrobial peptides in *Escherichia coli*: a review. Protein Expr Purif 80:260–267

Li T, Bin CX, Chen JC et al (2014a) Open and continuous fermentation: products, conditions and bioprocess economy. Biotechnol J 9:1503–1511

Li Y, Wang J, Yang J et al (2014b) Recombinant expression, purification and characterization of antimicrobial peptide ORBK in *Escherichia coli*. Protein Expr Purif 95:182–187

Li L, Mu L, Wang X et al (2017) A novel expression vector for the secretion of abaecin in *Bacillus subtilis*. Braz J Microbiol 48:809–814

Li H, Ali Z, Liu X et al (2019) Expression of recombinant tachyplesin I in *Pichia pastoris*. Protein Expr Purif 157:50–56

Li Z, Cheng Q, Guo H et al (2020) Expression of hybrid peptide EF-1 in *Pichia pastoris*, its purification, and antimicrobial characterization. Molecules 25:5538

Liang W, Diana J (2020) The dual role of antimicrobial peptides in autoimmunity. Front Immunol 11:1–9

Lima SMF, Freire MS, Gomes ALO et al (2017) Antimicrobial and immunomodulatory activity of host defense peptides, clavanins and LL-37, in vitro: an endodontic perspective. Peptides 95:16–24

Lin Q, Xie K, Chen D et al (2020) Expression and functional characterization of a novel antimicrobial peptide: human beta-defensin 118. Biomed Res Int 2020:1–10

Liscano Y, Oñate-Garzón J, Delgado JP (2020) Peptides with dual antimicrobial–anticancer activity: strategies to overcome peptide limitations and rational design of anticancer peptides. Molecules 25:4245

Liu SN, Han Y, Zhou ZJ (2011) Fusion expression of pedA gene to obtain biologically active pediocin PA-1 in *Escherichia coli*. J Zhejiang Univ Sci B 12:65–71

Liu H, Yang N, Mao R et al (2020) A new high-yielding antimicrobial peptide NZX and its antibacterial activity against *Staphylococcus hyicus* in vitro/vivo. Appl Microbiol Biotechnol 104:1555–1568

Lombardi L, Shi Y, Falanga A et al (2019) Enhancing the potency of antimicrobial peptides through molecular engineering and self-assembly. Biomacromolecules 20:1362–1374

Looser V, Bruhlmann B, Bumbak F et al (2015) Cultivation strategies to enhance productivity of *Pichia pastoris*: a review. Biotechnol Adv 33:1177–1193

Love KR, Dalvie NC, Love JC (2018) The yeast stands alone: the future of protein biologic production. Curr Opin Biotechnol 53:50–58

Mahlapuu M, Björn C, Ekblom J (2020) Antimicrobial peptides as therapeutic agents: opportunities and challenges. Crit Rev Biotechnol 40:978–992

Mauro VP, Chappell SA (2014) A critical analysis of codon optimization in human therapeutics. Trends Mol Med 20:604–613

Mayr LM, Bojanic D (2009) Novel trends in high-throughput screening. Curr Opin Pharmacol 9: 580–588

Mears L, Stocks SM, Sin G, Gernaey KV (2017) A review of control strategies for manipulating the feed rate in fed-batch fermentation processes. J Biotechnol 245:34–46

Meng DM, Lv YJ, Zhao JF et al (2018) Efficient production of a recombinant Venerupis philippinarum defensin (VpDef) in *Pichia pastoris* and characterization of its antibacterial activity and stability. Protein Expr Purif 147:78–84

Meng D-M, Li W-J, Shi L-Y et al (2019) Expression, purification and characterization of a recombinant antimicrobial peptide Hispidalin in *Pichia pastoris*. Protein Expr Purif 160:19–27

Mercer DK, Torres MDT, Duay SS et al (2020) Antimicrobial susceptibility testing of antimicrobial peptides to better predict efficacy. Front Cell Infect Microbiol 10:1–34

Mirski T, Niemcewicz M, Bartoszcze M et al (2017) Utilisation of peptides against microbial infections—a review. Ann Agric Environ Med 25:205–210

Mishra B, Reiling S, Zarena D, Wang G (2017) Host defense antimicrobial peptides as antibiotics: design and application strategies. Curr Opin Chem Biol 38:87–96

Møller TSB, Hay J, Saxton MJ et al (2017) Human β-defensin-2 production from *S. cerevisiae* using the repressible MET17 promoter. Microb Cell Factories 16:11

Moravej H, Moravej Z, Yazdanparast M et al (2018) Antimicrobial peptides: features, action, and their resistance mechanisms in bacteria. Microb Drug Resist 24:747–767

Moretta A, Salvia R, Scieuzo C et al (2020) A bioinformatic study of antimicrobial peptides identified in the Black Soldier Fly (BSF) *Hermetia illucens* (Diptera: Stratiomyidae). Sci Rep 10:16875

Moretta A, Scieuzo C, Petrone AM et al (2021) Antimicrobial peptides: a new hope in biomedical and pharmaceutical fields. Front Cell Infect Microbiol 11:1–26

Mulder KC, de Lima LA, Aguiar PS et al (2015) Production of a modified peptide clavanin in *Pichia pastoris*: cloning, expression, purification and in vitro activities. AMB Express 5:46

Naumov GI, Naumova ES, Boundy-Mills KL (2018) Description of *Komagataella mondaviorum* sp. nov., a new sibling species of *Komagataella* (*Pichia*) *pastoris*. Antonie van Leeuwenhoek 111:1197–1207

Nguyen LT, Haney EF, Vogel HJ (2011) The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol 29:464–472

Öztürk S, Ergün BG, Çalık P (2017) Double promoter expression systems for recombinant protein production by industrial microorganisms. Appl Microbiol Biotechnol 101:7459–7475

Pal G, Srivastava S (2015) Scaling up the production of recombinant antimicrobial Plantaricin E from a heterologous host, *Escherichia coli*. Probiotics Antimicrob Proteins 7:216–221

Pan X, Yang Y, Liu X et al (2016) Secretory expression of a heterologous protein, Aiio-AIO6BS, in *Bacillus subtilis* via a non-classical secretion pathway. Biochem Biophys Res Commun 478: 881–886

Parachin NS, Mulder KC, Viana AAB et al (2012) Expression systems for heterologous production of antimicrobial peptides. Peptides 38:446–456

Peebo K, Neubauer P (2018) Application of continuous culture methods to recombinant protein production in microorganisms. Microorganisms 6:56

Philip P, Meier K, Kern D et al (2017) Systematic evaluation of characteristics of the membrane-based fed-batch shake flask. Microb Cell Factories 16:122

Pieracci JP, Armando JW, Westoby M, Thommes J (2018) Industry review of cell separation and product harvesting methods. In: Biopharmaceutical processing. Elsevier, Amsterdam, pp 165–206

Potvin G, Ahmad A, Zhang Z (2012) Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. Biochem Eng J 64:91–105

Presnyak V, Alhusaini N, Chen YH et al (2015) Codon optimality is a major determinant of mRNA stability. Cell 160:1111–1124

Quinn GA, Maloy AP, McClean S et al (2012) Lipopeptide biosurfactants from *Paenibacillus polymyxa* inhibit single and mixed species biofilms. Biofouling 28:1151–1166

Raja Z, André S, Abbassi F et al (2017) Insight into the mechanism of action of temporin-SHa, a new broad-spectrum antiparasitic and antibacterial agent. PLoS One 12:e0174024

Ramirez LS, Pande J, Shekhtman A (2019) Helical structure of recombinant melittin. J Phys Chem B 123:356–368

Rathore AS, Agarwal H, Sharma AK et al (2015) Continuous processing for production of biopharmaceuticals. Prep Biochem Biotechnol 45:836–849

Rios AC, Moutinho CG, Pinto FC et al (2016) Alternatives to overcoming bacterial resistances: state-of-the-art. Microbiol Res 191:51–80

Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges. Front Microbiol 5:1–17

Rosano GL, Morales ES, Ceccarelli EA (2019) New tools for recombinant protein production in *Escherichia coli*: a 5-year update. Protein Sci 28:1412–1422

Sang M, Wei H, Zhang J et al (2017) Expression and characterization of the antimicrobial peptide ABP-dHC-cecropin A in the methylotrophic yeast *Pichia pastoris*. Protein Expr Purif 140:44–51

São Pedro MN, Silva TC, Patil R, Ottens M (2021) White paper on high-throughput process development for integrated continuous biomanufacturing. Biotechnol Bioeng 118:3275–3286

Schuller A, Cserjan-Puschmann M, Tauer C et al (2020) *Escherichia coli* σ70 promoters allow expression rate control at the cellular level in genome-integrated expression systems. Microb Cell Factories 19:58

Scott B, Wilcock A (2006) Process analytical technology in the pharmaceutical industry: a toolkit for continuous improvement. PDA J Pharm Sci Technol 60:17–53

Sen A, Kargar K, Akgün E, Plnar MC (2020) Codon optimization: a mathematical programing approach. Bioinformatics 36:4012–4020

Seyfi R, Kahaki FA, Ebrahimi T et al (2020) Antimicrobial peptides (AMPs): roles, functions and mechanism of action. Int J Pept Res Ther 26:1451–1463

Sezonov G, Joseleau-Petit D, D'Ari R (2007) *Escherichia coli* physiology in Luria-Bertani Broth. J Bacteriol 189:8746–8749

Shukla AA, Rameez S, Wolfe LS, Oien N (2017) High-throughput process development for biopharmaceuticals. In: Advances in biochemical engineering/biotechnology. Springer Nature, Berlin, pp 401–441

Silva ON, Mulder KCL, Barbosa AEAD et al (2011) Exploring the pharmacological potential of promiscuous host-defense peptides: from natural screenings to biotechnological applications. Front Microbiol 2:1–14

Silva TC, Eppink M, Ottens M (2021) Automation and miniaturization: enabling tools for fast, high-throughput process development in integrated continuous biomanufacturing. J Chem Technol Biotechnol jctb6792

Singh N, Pizzelli K, Romero JK et al (2013) Clarification of recombinant proteins from high cell density mammalian cell culture systems using new improved depth filters. Biotechnol Bioeng 110:1964–1972

Sinha R, Shukla P (2019) Antimicrobial peptides: recent insights on biotechnological interventions and future perspectives. Protein Pept Lett 26:79–87

Sousa DA, Mulder KCL, Nobre KS et al (2016) Production of a polar fish antimicrobial peptide in *Escherichia coli* using an ELP-intein tag. J Biotechnol 234:83–89

Taneda A, Asai K (2020) COSMO: a dynamic programming algorithm for multicriteria codon optimization. Comput Struct Biotechnol J 18:1811–1818

Tang X-S, Shao H, Li T-J et al (2012) Dietary supplementation with bovine lactoferrampin–lactoferricin produced by *Pichia pastoris* fed-batch fermentation affects intestinal microflora in weaned piglets. Appl Biochem Biotechnol 168:887–898

Tavares LS, Rettore JV, Freitas RM et al (2012) Antimicrobial activity of recombinant Pg-AMP1, a glycine-rich peptide from guava seeds. Peptides 37:294–300

Terpe K (2006) Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 72:211–222

Thapa RK, Diep DB, Tønnesen HH (2020) Topical antimicrobial peptide formulations for wound healing: current developments and future prospects. Acta Biomater 103:52–67

Tian ZG, Teng D, Yang YL et al (2007) Multimerization and fusion expression of bovine lactoferricin derivative LfcinB15-W4,10 in *Escherichia coli*. Appl Microbiol Biotechnol 75: 117–124

Tian J, Yan Y, Yue Q et al (2017) Predicting synonymous codon usage and optimizing the heterologous gene for expression in *E. coli*. Sci Rep 7:9926

Tian L, Zhang D, Su P et al (2019) Design, recombinant expression, and antibacterial activity of a novel hybrid magainin–thanatin antimicrobial peptide. Prep Biochem Biotechnol 49:427–434

Tornesello AL, Borrelli A, Buonaguro L et al (2020) Antimicrobial peptides as anticancer agents: functional properties and biological activities. Molecules 25:2850

Tripathi NK, Shrivastava A (2019) Recent developments in bioprocessing of recombinant proteins: expression hosts and process development. Front Bioeng Biotechnol 7:1–35

Viel JH, Jaarsma AH, Kuipers OP (2021) Heterologous expression of mersacidin in *Escherichia coli* elucidates the mode of leader processing. ACS Synth Biol 10:600–608

Vilas Boas LCP, de Lima LMP, Migliolo L et al (2017) Linear antimicrobial peptides with activity against herpes simplex virus 1 and Aichi virus. Pept Sci 108:e22871

Vogt CM, Schraner EM, Aguilar C, Eichwald C (2016) Heterologous expression of antigenic peptides in *Bacillus subtilis* biofilms. Microb Cell Factories 15:137

Wade HM, Darling LEO, Elmore DE (2019) Hybrids made from antimicrobial peptides with different mechanisms of action show enhanced membrane permeabilization. Biochim Biophys Acta Biomembr 1861:182980

Wang A, Wang S, Shen M et al (2009) High level expression and purification of bioactive human α-defensin 5 mature peptide in *Pichia pastoris*. Appl Microbiol Biotechnol 84:877–884

Wang FJ, Song HL, Wang XM et al (2012) Tandem multimer expression and preparation of hypoglycemic peptide MC6 from *Momordica charantia* in *Escherichia coli*. Appl Biochem Biotechnol 166:612–619

Wang J, Dou X, Song J et al (2019) Antimicrobial peptides: promising alternatives in the post feeding antibiotic era. Med Res Rev 39:831–859

Weinacker D, Rabert C, Zepeda AB et al (2013) Applications of recombinant *Pichia pastoris* in the healthcare industry. Braz J Microbiol 44:1043–1048

Whitesides GM (2006) The origins and the future of microfluidics. Nature 442:368–373

Wibowo D, Zhao C-X (2019) Recent achievements and perspectives for large-scale recombinant production of antimicrobial peptides. Appl Microbiol Biotechnol 103:659–671

Wu R, Wang Q, Zheng Z et al (2014) Design, characterization and expression of a novel hybrid peptides melittin (1-13)-LL37 (17-30). Mol Biol Rep 41:4163–4169

Xiao S, Gao Y, Wang X et al (2017) Peroxisome-targeted and tandem repeat multimer expressions of human antimicrobial peptide LL37 in *Pichia pastoris*. Prep Biochem Biotechnol 47:229–235

Xu X, Jin F, Yu X et al (2007) High-level expression of the recombinant hybrid peptide cecropinA (1-8)-magainin2(1-12) with an ubiquitin fusion partner in *Escherichia coli*. Protein Expr Purif 55:175–182

Yang N, Teng D, Mao R et al (2019) A recombinant fungal defensin-like peptide-P2 combats multidrug-resistant *Staphylococcus aureus* and biofilms. Appl Microbiol Biotechnol 103:5193–5213

Yang Y, Wu D, Wang C et al (2020) Hybridization with insect cecropin a (1–8) improve the stability and selectivity of naturally occurring peptides. Int J Mol Sci 21:1470

Ÿztürk S, Ÿalık P, Ÿzdamar TH (2016) Fed-batch biomolecule production by *Bacillus subtilis*: a state of the art review. Trends Biotechnol 34:329–345

Zandsalimi F, Talaei S, Noormohammad Ahari M et al (2020) Antimicrobial peptides: a promising strategy for lung cancer drug discovery? Expert Opin Drug Discov 15:1343–1354

Zhan N, Zhang L, Yang H et al (2021) Design and heterologous expression of a novel dimeric LL37 variant in *Pichia pastoris*. Microb Cell Factories 20:143

Zhang J, Movahedi A, Wei Z et al (2016) High-level SUMO-mediated fusion expression of ABP-dHC-cecropin A from multiple joined genes in *Escherichia coli*. Anal Biochem 509:15–23

Zhang K, Su L, Duan X et al (2017) High-level extracellular protein production in *Bacillus subtilis* using an optimized dual-promoter expression system. Microb Cell Factories 16:32

Zhang X, Jiang A, Qi B et al (2018) Secretion expression of human neutrophil peptide 1 (HNP1) in *Pichia pastoris* and its functional analysis against antibiotic-resistant *Helicobacter pylori*. Appl Microbiol Biotechnol 102:4817–4827

Zhang L, Wei D, Zhan N et al (2020) Heterologous expression of the novel α-helical hybrid peptide PR-FO in *Bacillus subtilis*. Bioprocess Biosyst Eng 43:1619–1627

Zhao W, Wang J, Deng R, Wang X (2008) Scale-up fermentation of recombinant *Candida rugosa* lipase expressed in *Pichia pastoris* using the GAP promoter. J Ind Microbiol Biotechnol 35: 189–195

Zhao H, Tang J, Cao L et al (2015) Characterization of bioactive recombinant antimicrobial peptide parasin I fused with human lysozyme expressed in the yeast *Pichia pastoris* system. Enzym Microb Technol 77:61–67

Zhou Y, Cao W, Wang J et al (2005) Comparison of expression of monomeric and multimeric adenoregulin genes in *Escherichia coli* and *Pichia pastoris*. Protein Pept Lett 12:349–355

Zhoua Z, Danga Y, Zhou M et al (2016) Codon usage is an important determinant of gene expression levels largely through its effects on transcription. Proc Natl Acad Sci U S A 113: E6117–E6125

Zobel S, Kumpfmüller J, Süssmuth RD, Schweder T (2015) *Bacillus subtilis* as heterologous host for the secretory production of the non-ribosomal cyclodepsipeptide enniatin. Appl Microbiol Biotechnol 99:681–691

Zydney AL (2016) Continuous downstream processing for high value biological products: a review. Biotechnol Bioeng 113:465–475