BioMed Research International

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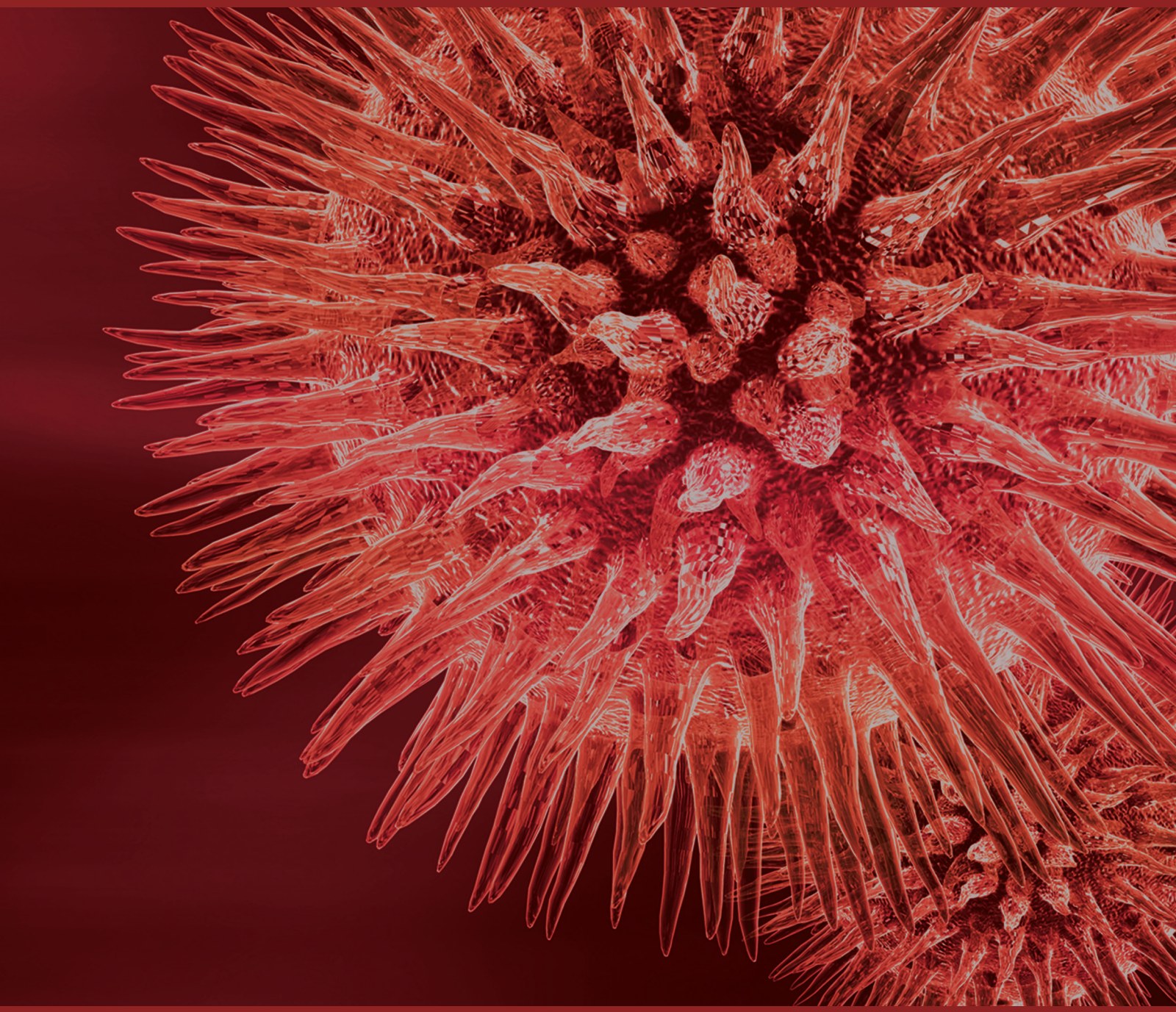
Guest Editors: Flavia Marinelli, Olga Genilloud, Victor Fedorenko, and Eliora Z. Ron



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Editorial

Specialized Bioactive Microbial Metabolites: From Gene to Product

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Natural products continue to play an important role in the discovery of new therapeutic candidates. Over the past 30 years, natural products or their derivatives have accounted for 60% of new anticancer agents and almost 75% of all new antibacterial molecules. One hundred natural products and natural product-derived substances were being evaluated in clinical trials or were being registered at the end of 2013. Bioactive molecules have been isolated from many terrestrial and marine organisms, including plants, marine invertebrates, and microorganisms, the latter being the source selected more often for pharmaceutical drug discovery programs. Microorganisms (traditionally filamentous actinobacteria and fungi, but more recently cyanobacteria, microalgae, and myxobacteria) are one of the most prolific sources among living organisms for the production of bioactive molecules. Exploitation of their specialized (commonly named secondary) metabolism has guaranteed for decades the discovery of novel antibiotics and other compounds with unprecedented chemical characteristics and biological properties not existing in screening libraries of synthetic compounds.

Despite the lack of big pharma interest in addressing the topic in the last decade, microbial products continue to represent today one of the most interesting sources for the discovery of novel drugs and research in the field is currently benefiting from progress that has been made in other related fields (microbial ecology, metagenomics, metabolomics, or synthetic biology), fields which have provided a deeper understanding of the microbiome and thus the development of new tools to foster the discovery of novel compounds. A wealth of microbial gene clusters that encode novel biosynthetic pathways (or interesting variants of those already described) to bioactive microbial products is being unveiled with the ever increasing number of sequenced microbial genomes. Whereas the difficulty to discover and develop in a reasonable time and acceptable cost new products from microbial sources has been widely recognized, recent advances in gene mining and heterologous expression, knowledge on regulatory networks, new analytical deconvolution, and chemical characterization tools are opening new avenues in the field of microbial product discovery.

This special issue includes five papers (three reviews and two research papers) addressing diverse aspects related to the understanding and eventually facing the current bottlenecks in the process of microbial product discovery and development.

One of the papers is a review tracing the status on antibacterial discovery and development from microbial sources. Nowadays we are all aware that bacterial resistance to all currently used antibiotics has emerged for both Gram-positive and Gram-negative bacteria. This threatening situation urgently calls for a concerted international effort among governments, the pharmaceutical industry, biotechnology companies, and the academic world to react and support the development of new antibacterial agents. The authors of this review, which include the editors of this special issue, after an introduction of the medical needs and the mechanisms of antibacterial resistance, investigate those

screening ingredients (i.e., how to build microbial product libraries, methods for cultivation and extraction, the need of chemical dereplication for an early elimination of already known molecules, and tools for strain selection) that are nowadays crucial for discovering novel antibacterials. An overview of the current fermentation technology used to produce specialized metabolites and a detailed analysis of the chances for genetically improving the producing microbes in the postgenomics era are following. For the majority of antibiotics, including those recently marketed, the only feasible supply process continues to be fermentation, total synthesis being too complicated or too expensive. Thus, manipulating and improving microbial strains and their growing conditions remain the main tools to reduce production volumes and costs and guarantee quality and reproducibility of the drug bulks.

A second review by researchers from Brazil is focussing on microalgae diversity exploitation for discovering and producing interesting specialized metabolites endowed with anti-inflammatory, antimicrobial, and antioxidant activities. Microalgae are microorganisms that have different morphological, physiological, and genetic traits: they include prokaryotic (cyanobacteria) and eukaryotic organisms. Among the thousands of species of microalgae believed to exist, only a small number are stored in collections around the world, and it is estimated that only a few hundred are investigated for interesting compounds present in their biomass. After an analysis of the product potentialities of genera such as *Nostoc*, *Spirulina*, *Chlorella,* and *Dunaliella*, advantages and limits of their cultivation and extraction are investigated. Due to their photoautotrophic metabolism, microalgal cultivation processes need to be better understood: microalgae can become an environmentally friendly and economically viable source of compounds of interest, once their production is optimized in a controlled culture and properly constructed bioreactors.

The third review written by South Korean researchers discusses the recent trends in the research and production of violacein, which is a purple pigment produced by both natural and genetically modified bacterial strains. The bisindole violacein is formed by the condensation of two tryptophan molecules through the action of five proteins. The genes required for its production, *vioABCDE*, and the regulatory mechanisms employed have been studied within a small number of violacein producing strains. As a compound, violacein is known to have diverse biological activities, including as an anticancer agent and as an antibiotic against *Staphylococcus aureus* and other Gram-positive pathogens. Identifying the biological roles of this pigmented molecule is of particular interest, and understanding violacein's function and mechanism of action has relevance to those unmasking any of its commercial or therapeutic benefits. As usually happens with specialized metabolites, the production of violacein and its related derivatives is strictly regulated and its production is limited. To face this production bottleneck, various groups are seeking to improve the fermentative yields of violacein through genetic engineering and synthetic biology.

The two research papers completing the issue are brilliant examples of what was anticipated within the reviews as critical steps in the discovery and development of novel specialized metabolites. Interestingly, both of them are on lantibiotics, which represent an attractive option of a new class of molecules that might overcome arising resistance. Lantibiotics are ribosomally synthetized and posttranslationally modified peptides possessing potent antimicrobial activity against aerobic and anaerobic Gram-positive pathogens, including those increasingly resistant to β-lactams and glycopeptides. For some of them, a specific mode of action inhibiting cell wall biosynthesis (not antagonized by vancomycin) has been demonstrated, explaining the renewed interest for such chemical class of antibacterial peptides.

The paper published by the Italian group deals with an example of an efficient strategy for lantibiotic screening applied to 240 members of a newly described genus of filamentous actinomycetes, named *Actinoallomurus*, which is considered a yet-poorly exploited promising source for novel bioactive metabolites. By combining antimicrobial differential assay against *Staphylococcus aureus* and its L-form (also in the presence of a β-lactamase cocktail or Ac-Lys-D-alanyl-D-alanine tripeptide), with LC-UV-MS dereplication coupled with bioautography and database query, a novel producer of the potent microbisporicin complex was rapidly identified. Beside the interest in characterizing this novel producer of microbisporicin, this paper drives the attention to the relevance of the process termed dereplication, that is, the process of distinguishing those microbial extracts that contain known bioactive metabolites from those that contain novel compounds of interest, saving resources and speeding up the discovery process of novel drugs.

Finally, the last paper by a research group from China highlights the need to improve the fermentation conditions to sustain sublancin 168 production by a strain of *Bacillus subtilis*. Fermentation is the favourite way to produce this antimicrobial peptide, but the authors claim that the low yield of this stable lantibiotic, that has a broad spectrum of antimicrobial activity, has constrained its commercial application. In this specific case, the authors first screen carbon and nitrogen sources to identify key medium ingredients and then develop an experiment design approach to optimize chemical composition of the cultivation medium and temperature of incubation. The volumetric antimicrobial peptide productivity was double and the study envisages further increments that might be achieved following the developed model.

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Research Article

A Novel Microbisporicin Producer Identified by Early Dereplication during Lantibiotic Screening

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With the increasing need of effective antibiotics against multi-drug resistant pathogens, lantibiotics are an attractive option of a new class of molecules. They are ribosomally synthetized and posttranslationally modified peptides possessing potent antimicrobial activity against aerobic and anaerobic Gram-positive pathogens, including those increasingly resistant to β-lactams and glycopeptides. Some of them (actagardine, mersacidin, planosporicin, and microbisporicin) inhibit cell wall biosynthesis in pathogens and their effect is not antagonized by vancomycin. Hereby, we apply an efficient strategy for lantibiotic screening to 240 members of a newly described genus of filamentous actinomycetes, named *Actinoallomurus*, that is considered a yet-poorly-exploited promising source for novel bioactive metabolites. By combining antimicrobial differential assay against *Staphylococcus aureus* and its L-form (also in the presence of a β-lactamase cocktail or Ac-Lys-D-alanyl-D-alanine tripeptide), with LC-UV-MS dereplication coupled with bioautography, a novel producer of the potent microbisporicin complex was rapidly identified. Under the commercial name of NAI-107, it is currently in late preclinical phase for the treatment of multi-drug resistant Gram-positive pathogens. To our knowledge, this is the first report on a lantibiotic produced by an *Actinoallomurus* sp. and on a microbisporicin producer not belonging to the *Microbispora* genus.

1. Introduction

Lantibiotics, the abbreviation for "lanthionine containing antibiotics," are a class of ribosomally synthetized and post-translationally modified peptides produced by and active versus Gram-positive bacteria [1, 2]. They are characterized by the thioether-containing linkages lanthionine (Lan) and/or methyllanthionine (MeLan), originating by the dehydration of Ser/Thr residues in a precursor peptide followed by intramolecular addition of Cys to the dehydrated residues. Nisin, the best characterized lantibiotic, has been used as a food preservative to combat food-borne pathogens for more than forty years without the development of widespread antibiotic resistance [3]. As such, lantibiotics are a promising group of natural products to battle the continuous rise of antibiotic resistance [4]. Some of them like actagardine [5], mersacidin [6], planosporicin [7], and microbisporicin [8] possess potent antimicrobial activity against aerobic and anaerobic Gram-positive pathogens, including those increasingly resistant to β-lactams and glycopeptides [9]. They inhibit cell wall biosynthesis [10] without showing cross-resistance with vancomycin [11]. Furthermore, lantibiotics have been shown to have promising efficacy and pharmacokinetics in animal models [12, 13].

The renewed interest for this class of specialized microbial metabolites has prompted in the last decade the search of novel lantibiotics following different approaches: (i) by chemical modification of known molecules [14]; (ii) by gene site-directed mutagenesis and expression of lantibiotics' variants in heterologous hosts [15–17]; (iii) by screening untapped microbial diversity for novel scaffolds [7, 8, 18]. It is widely recognized that the success of the last approach depends

mostly on the novelty of the microbial sources and on the selectivity of the screening strategy [19, 20]. Presently, after decades of massive natural product screening, one of the limiting hindrance is the reisolation of already discovered bioactive molecules [21]. Since structure elucidation of a natural product purified from a complex matrix such as microbial extract is a demanding step, early identification of known or undesirable compounds, hereby indicated as dereplication, is a key activity in microbial natural product screening, saving resources and speeding up the discovery process of novel drugs [19–22].

In this work, we combine a robust and selective lantibiotic screening strategy applied to a newly described genus of filamentous actinomycetes named *Actinoallomurus* [23] with an early procedure of dereplication. Recent papers claim that *Actinoallomurus* is a good source of novel bioactive metabolites [24, 25], but to our knowledge it has not been yet exploited for the production of lantibiotics.

2. Materials and Methods

2.1. Bacterial Strains. *Staphylococcus aureus* 209 ATCC 6538P (L100) were purchased from the American Type Culture Collection (ATCC; Manassas VA). L-form cells (L3751) were prepared from L100 by exposure to 100 U of penicillin in Enterococcal Brain Heart Infusion/S (EBH/S) supplemented with 5% NaCl, 5% sucrose, and 10% horse serum as previously described [5, 26]. L-forms were then cultured on similarly supplemented brain heart infusion agar containing no antibiotic. *S. aureus* Smith ATCC19636 (L819), *Streptococcus pyogenes* C203 ATCC12384 (L49), and other clinical isolates (*S. aureus* L1400, *Enterococcus faecalis* L559, *Enterococcus faecalis* Van A L560, *Escherichia coli* SKF12140 L47, and *Candida albicans* SKF2270 L145) were maintained in the Fondazione Istituto Insubrico Ricerca per la Vita (F.I.I.R.V.) culture collection (L collection) at Gerenzano, Italy.

2.2. Media and Culture Conditions. *Actinoallomurus* spp. were isolated from different soil sources with the following method: 250 mg finely ground and dried soil (100°C for 60 min) was poured onto agar plates of HSA5.5 medium (in g/L: humic acid, 2 previously dissolved in 10 mL 0.2 NaOH aqueous solution; $FeSO_4 \cdot 7H_2O$, 0.001; $MnCl_2 \cdot 4H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; $NiSO_4 \cdot 6H_2O$, 0.001; MES, 2; agar, 20; add 1 mL CMM vitamin solution containing 25 μg thiamin hydrochloride, 250 μg calcium pantothenate, 250 μg nicotinic acid, 500 μg mg biotin, 1,25 mg riboflavin, 6 μg vitamin B_{12}, 25 μg *p*-aminobenzoic acid, 500 μg folic acid, and 500 μg pyridoxal hydrochloride; pH adjusted to 5.5 before sterilization). All the medium components were purchased from Sigma-Aldrich, unless otherwise stated. Isolation plates were incubated at 50°C for 24 h and then at 28°C for more than four weeks. Pure colonies were picked up, checked at the microscope, and then maintained at 28°C on pH 5.5 ISP3 agar plates. Morphology was observed at the stereoscope (Zeiss) and at the light microscope (model ULWD-CDPlan; Olympus) fitted with a 3CCD camera (Sony). For liquid cultures, a loopful of mycelium was scrapped off and transferred in a 80 mL baffled Erlenmeyer flask containing 15 mL of AF5 (g/L: dextrose, 20; yeast extract, 2; soybean meal, 8; NaCl, 1; and MES, 10; pH adjusted to 5.5 before sterilization) or M85.5 (g/L: dextrose, 10; yeast extract, 2; beef extract 2; starch, 20; casein hydrolysate, 2; and MES, 20; pH adjusted to 5.5 before sterilization). Unless otherwise stated, all fermentation medium components were from Constantino, Arese, Italy. After six days, 10% (v/v) of the culture was transferred into 500 mL flasks containing 100 mL of AF5 or M85.5. Flasks were incubated for 16–18 days at 28°C on a rotary shaker at 200 rpm. After centrifugation at 3000 rpm for 15 min, broths (10 mL) were extracted by adding 2.3% (v/v) polystyrenic resin HP-20 (Mitsubishi Chemical Co.) and eluting it batchwise with 5 mL pure methanol (screening broth extracts). For the preparation of a partially purified fraction (crude extract), the strain was grown as reported above in 1000 mL flasks containing 350 mL AF5 medium. Approximately, 300 mL broth was loaded on HP-20 resin (7.5 mL) that was eluted stepwise by increasing the organic phase percentage: first by 30 mL of methanol : water 2 : 3 (v/v), then by 30 mL methanol : water 4 : 1 (v/v), and finally by 30 mL methanol : isopropanol 9 : 1 (v/v). The last eluted fraction was concentrated to dryness in rotavapor. Preparative chromatography was followed by UV spectroscopy and bioactivity (see below). Mycelium extracts were prepared by directly adding 2 mL ethanol per gram wet mycelium; samples were shaken at 200 rpm for 2 h. The organic phases were finally concentrated to dryness under a N_2 flow in a Turbo-Vap unit and stored at −10°C.

2.3. Lantibiotic Screening Differential Assay. Broth and mycelium screening extracts from the F.I.I.R.V. collection of *Actinoallomurus* strains isolated according as above were screened in liquid microplate assays for their antimicrobial activity on *S. aureus* 209 ATCC 6538P (L100) and to its L-form cells (L3751), as described in detail in [7]. In brief, *S. aureus* 209 ATCC 6538P (L100) and its L-form cells (L3751) were maintained at −80°C in Nutrient Broth (Difco) to which 20% (v/v) glycerol was added. EBH/S supplemented with 5% (v/v) horse serum was used as medium. For the wild-type inoculum, 10 μL of extracts previously dissolved in DMSO : H_2O 1 : 9 (v/v) were added to 1×10^5 CFU/mL in 90 μL of culture broth. For L-form cells, aliquots of liquid cultures grown overnight in EBHI/S to $O.D._{620\,nm} = 0.2$ were used as inoculum. Incubation time was 24 h at 35°C in air, and then growth inhibition was measured at $O.D._{620\,nm}$. Reference actagardine, planosporicin, microbisporicin, mersacidin, and nisin standards were used [7, 8] and MIC levels were determined by broth microdilution assay as recommended by the National Committee for Clinical Laboratory Standards [27]. To identify β-lactam producers, antimicrobial activity versus *S. aureus* 209 ATCC 6538P (L100) was measured in a liquid microplate assay after adding the following cocktail of β-lactamases: Penicillase Type I from *Bacillus cereus* (Sigma P0389), 0.001 U/mL; Penicillase Type II from *Bacillus cereus* (Sigma P6018), 0.002 U/mL; Penicillase type III from *Enterobacter cloacae* (Sigma P4399), 0.0025 U/mL; and Penicillase type IV from *Enterobacter cloacae* (Sigma P4524), 0.5 U/mL.

To identify glycopeptide producers, antimicrobial activity versus *S. aureus* 209 ATCC 6538P (L100) was measured in a liquid microplate assay after adding 2 mg/mL of Ac-Lys-D-alanyl-D-alanine (Chem-Impex International Inc., IL).

2.4. LC-UV-MS and MS/MS Analyses. LC-MS and MS/MS experiments were performed in a ThermoQuest Finnigan LCQ Advantage mass detector equipped with an ESI interface and Thermo Finnigan Surveyor MS pump, photo diode array detector (PDA) (UV6000; Thermo Finnigan), and an autosampler. The Thermo Surveyor HPLC instrument was equipped with a Symmetry C18 (5 μm, 4,6 × 250 mm Waters Chromathography) column. Analyses were performed at 1 mL/min flow rate according to a multistep linear gradient using phase B (acetonitrile) in phase A (acetonitrile: 10 mM ammonium formiate pH 4.5 buffer, 5 : 95 v/v). The column was equilibrated in 20% phase B; after 1 min in these conditions, the concentration of phase B increased up to 90% in 31 min, followed by further 4 min at 90% phase B. Full UV-visible spectra of the eluted molecules, 200–600 nm range, were detected by PDA. MS spectra were obtained by electrospray ionization, both in positive and in negative mode. MS/MS were performed on the same apparatus by changing ionization energy both in positive and negative mode. The ThermoQuest Finnigan LCQ Advantage mass detector was previously tuned and calibrated in electrospray mode in the following conditions: Spray Voltage: 4.5 kV; Capillary temperature: 220°C; Capillary Voltage: 3 V. LC/MS/MS were performed on the same apparatus in dependent scan mode, mass range 900–1200, default charge state 2, and enabling charge screening, using a normalized collision energy (CID) of 30 ev, Act Q 0.250 Act TIME (ms) 30.

For bioautography, fractions (1 mL, eluting at 1 mL/min) from the HPLC column were collected, dried, and resuspended in 100 μL aqueous solution at 10% (v/v) DMSO. 10 μL were tested for antimicrobial activity. UV and mass spectra of molecules present in the active fractions were compared with those collected in the ABL database, which contains data on approximately 30,000 microbial metabolites collected from literature and patents since 1950 [20, 28], and in the commercially available Antibase (http://wwwuser.gwdg.de/~hlaatsc/antibase.htm).

2.5. Antimicrobial Activity. Antimicrobial activity was determined by broth microdilution assay according to standard guidelines [27]. The growth media utilized to determine the MIC were cation-adjusted Difco Mueller Hinton Broth (MHB) for *Staphylococci*, *Enterococci*, and *E. coli*, Todd Hewitt Broth (THB) for *Streptococci*, and RPMI-1640 medium (RPMI) for *C. albicans*. Typically, a twofold serial dilution of the test compound was performed in a sterile 96-well microplate inoculated with 10^4 CFU/mL of the test strain in the appropriate medium. The microplate was then incubated for 18–24 h at 35°C. The MIC was determined by visual examination of the microplates with the aid of a magnifying mirror as the lowest concentration of antibiotic that showed no visible sign of microbial growth.

2.6. 16S rRNA Gene Sequencing. Genomic DNA was extracted with the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) by colony picking; PCR-mediated amplification of the 16S rRNA gene, purification of the PCR products and sequencing were carried out as previously described [29]. Alignments of 16S rRNA gene sequences were conducted with BLASTN (http://www.ncbi.nlm.nih.gov/blast/). For the construction of the phylogenetic tree, selected sequences were aligned with Clustal-Omega (from the EMBL-EBI site) and analyzed with BioEdit [30]. Distance matrices were calculated with MEGA5.2, using the Maximum Likelihood method implemented in the program and the method of Jukes and Cantor. Trees were inferred using the Nearest-Neighbor-Interchange (NNI) heuristic method and making the initial tree with both Neighbour Joining and BioNJ, and selecting the superior tree (all methods are included in the MEGA package). All analyses were performed on a bootstrapped data set containing 500 replicates.

3. Results and Discussion

3.1. Lantibiotic Screening of Actinoallomurus spp. 880 extracts were obtained from broth and mycelium of 240 *Actinoallomurus* spp. (from the F.I.I.R.V. collection) isolated as described in Section 2, after six days of growth in fermentation media AF5 and M85.5. Primary screening was based on the differential activity assay versus *S. aureus* and its L-form. L-forms are protoplast-type cells derived from *S. aureus* that are able to replicate in appropriate osmotic conditions despite the lack of a functional cell wall [5, 7, 26]. As previously shown in [7], L-forms are equally or more sensitive than parental cells to those antibiotics acting on molecular targets other than cell wall biosynthesis. They are indeed resistant to peptidoglycan synthesis inhibitors. Extracts from 67 strains were equally active on *S. aureus* and its L-form, whereas only 2 strains gave a significant level of differential activity: their MICs versus L-form cells were at least eightfold higher than those against the whole cells. Secondary selection was based on whether antimicrobial activity against *S. aureus* could be reversed by a β-lactamase cocktail or by adding Ac-Lys-D-alanyl-D-alanine tripeptide, which mimics the glycopeptide cell target. This step was introduced to eliminate PG inhibitors belonging to the known classes of β-lactams and glycopeptides. Only one strain (named F31/11) passed the secondary selection: its activity versus *S. aureus* was not abolished by adding either the β-lactamase cocktail or the Ac-Lys-D-alanyl-D-alanine tripeptide. F31/11 antimicrobial activity was reconfirmed upon its repeated fermentation, and it was found to be excreted into the medium (Table 1) as well as being associated to the mycelium (data not shown). Both extracts were found active against clinical isolates representative of Gram-positive pathogens, including one methicillin resistant *S. aureus* (MRSA) and one vancomycin-resistant *E. faecalis* (VanA). The Gram-negative *E. coli* was insensitive and, consistent with the mode of action of bacterial cell wall inhibitors, no activity was observed against *S. aureus* L-form (L3751) and the eukaryote *C. albicans*.

Table 1: Antimicrobial activity of the screening extract from F31/11 broth measured as an endpoint in microdilution method, that is, the highest dilution that inhibits 80% of test strain growth.

Microorganism	Medium	Active dilution
L100 *S. aureus* ATCC 6538P	EBH/S	>1 : 64
L3751 *S. aureus* L-form	EBH/S	<1 : 4
L100 *S. aureus* ATCC 6538P	EBH/S + β-lactamase cocktail	1 : 64
L100 *S. aureus* ATCC 6538P	EBH/S + Ac-Lys-D-Ala-D-Ala	1 : 64
L1400 *S. aureus* MRSA	MHB	1 : 64
L49 *S. pyogenes*	THB	>1 : 64
L559 *E. faecalis*	MHB	1 : 8
L560 *E. faecalis* Van A	MHB	1 : 16
L47 *E. coli*	MHB	<1 : 4
L145 *C. albicans*	RPMI	<1 : 4

Table 2: Antimicrobial activity of F31/11 crude extract in comparison to planosporicin, actagardine, microbisporicin, mersacidin, and nisin standards. MICs were determined by broth microdilution assay [27].

Strain	MIC (mg/L)					
	Planosporicin	Actagardine	Microbisporicin	Mersacidin	Nisin	F31/11
L100 *S. aureus* ATCC6538P	2	32	≤0.13	4	0.5	4
L3751 *S. aureus* L-form	>128	>128	>128	64	16	>128
L1400 *S. aureus* MRSA	16	16	≤0.13	8	2	8
L49 *S. pyogenes*	<1	2	<1	n.d	n.d	1
L47 *E. coli*	>128	>128	>128	n.d	>128	>128
L145 *C. albicans*	>128	>128	>128	n.d	>128	>128

3.2. Antimicrobial Activity of F31/11. The pattern of antimicrobial activity of F31/11 extract shown in Table 1 matches with the one expected for a potent lantibiotic. To confirm this, we prepared an enriched crude extract as described in Section 2 by partition chromatography from F31/11 broth, which was tested in parallel with standard samples of lantibiotics (actagardine, planosporicin, microbisporicin, mersacidin, and nisin). Data reported in Table 2 confirm the antimicrobial potency of the unknown antibiotic produced by F31/11.

3.3. LC-UV-MS Coupled with Bioautography. UV and MS spectra were simultaneously collected during HPLC chromatography fractionation and each chromatographic fraction was in parallel tested for antimicrobial activity versus *S. aureus*, its L-form and versus a MRSA clinical isolate, conducting the so called bioautography (Figure 1). Figure 1(a) shows the presence of many compounds in the MS-HPLC profile by electrospray ionization, both in positive and in negative mode, within the crude extract from F31/11. Fractionation coupled with the activity profile shown in Figure 1(b) indicates a major peak eluting at *ca*. 11.7 min (−ESI) and 11.6 (+ESI), which corresponds to the putative lantibiotic, which inhibits the microbial growth of *S. aureus*, but not its L-form. Base peak ion extraction pointed out that the molecule eluting at 11.7 min has *m/z* of 1115.2 in negative mode (−ESI) and of 1117.2 in positive mode (+ESI). MS spectrum (Figure 1(c)) shows that the lowest molecular weight signals correspond to double charged species, more exactly to the double-charged ion $[M + 2H]^{2+}$ at *m/z* of 1117.2, $[M + Na + H]^{2+}$ at *m/z* of 1126.1, and $[M - 2H]^{2-}$ at *m/z* 1115.2, suggesting a molecular weight of 2230 Da. As shown in Figure 1S in Supplementary Material available online at http://dx.doi.org/10.1155/2015/419383, the full scan mass spectrum range of 1000–3000 mass units value of this peak shows the presence of the signal corresponding to the single-charged ion $[M + H]^{+}$ at *m/z* of 2231.2. The UV spectrum shows two shoulders at 225 and 267 nm (Figure 1(d)).

The bioautography of the mycelium extract led to the identification of the same molecular species eluting at 11.7 min and highlighted the presence of a second peak eluting at 12.2 min. This peak was also present (but in lower amount) in the LC/MS profile from the broth extract (Figure 1(a)). This last peak shows a similar UV profile as the one at 11.7 min, showing two shoulders at 226 and 267 nm (Figure 1(f)). It is characterized by a double-charged ion $[M + 2H]^{2+}$ at *m/z* of 1125.3, a double-charged ion $[M + Na + H]^{2+}$ at *m/z* 1136.2 in positive current ion, and a signal corresponding to the double-charged ion $[M - 2H]^{2-}$ at *m/z* of 1123.4 in the negative mode (Figure 1(e)). As shown in Figure 1S in Supplementary Material, the full scan mass spectrum range of 1000–3000 mass units value of this peak shows the presence of the signal corresponding to the single-charged ion $[M + H]^{+}$ at *m/z* of 2247.2.

To gain further information on the structure of the two active compounds eluting at 11.7 and 12.2 min, we investigated them by further runs of LC/MS/MS: the signal corresponding to *m/z* of 1117.2 originated an intense peak at *m/z* of 1099.54, while in the same conditions the signal

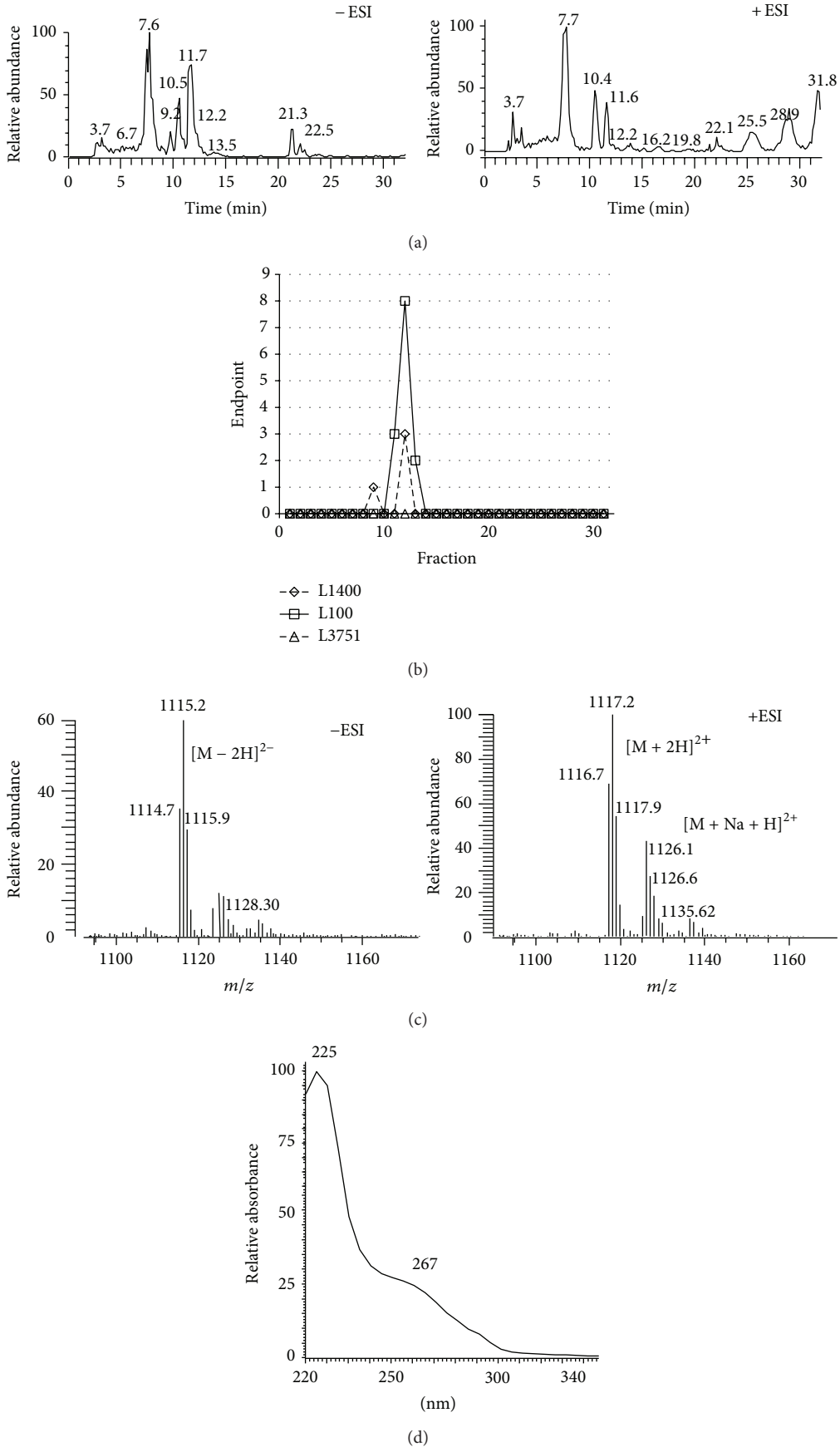

Figure 1: Continued.

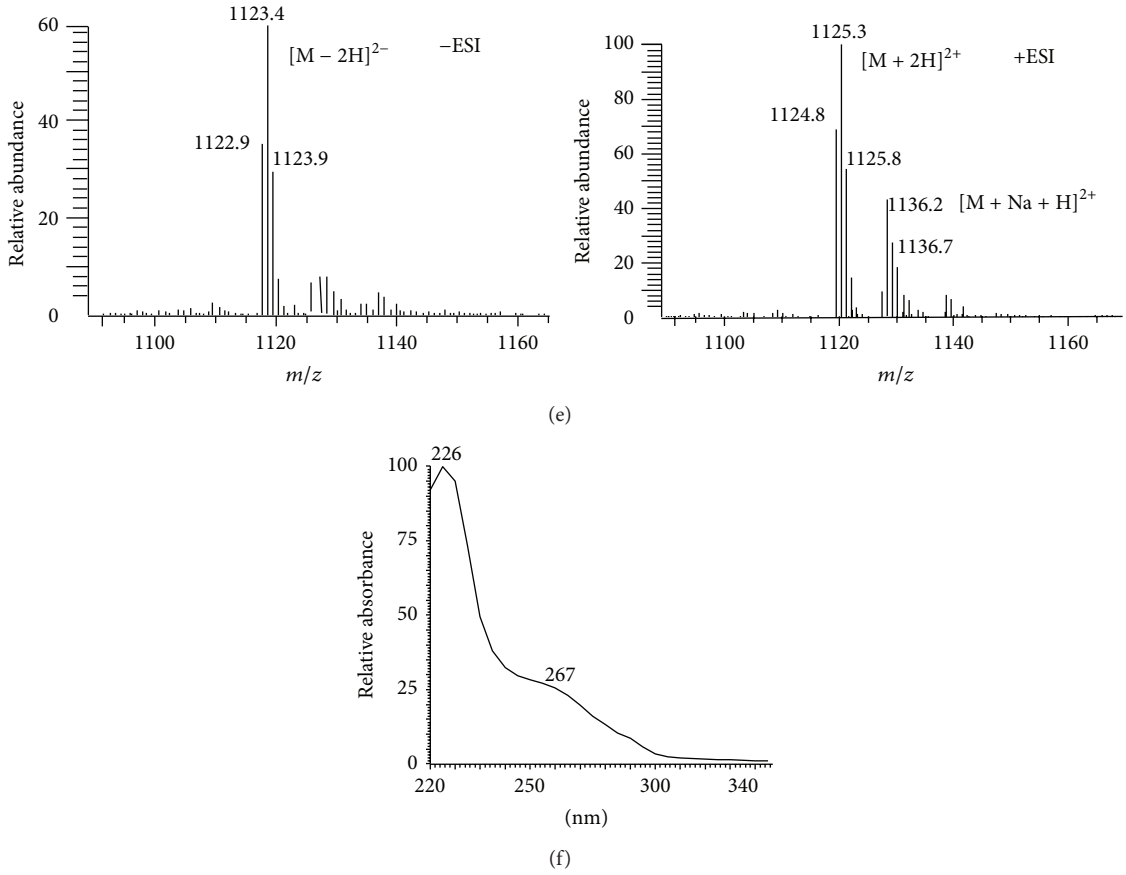

Figure 1: MS-HPLC profiles of the F31/11 broth screening extract: (a) MS trace in negative and positive mode; (b) bioautography: each HPLC fraction was tested versus *S. aureus* MRSA L1400, MSSA L100, and L-form L3751 in dose dilution; (c) MS spectrum of the peak eluting at 11.7 min in negative and positive mode; (d) UV spectrum of the peak eluting at 11.7 min; (e) MS spectrum of the peak eluting at 12.2 min in negative and positive mode; (f) UV spectrum of the peak eluting at 12.2 min. In UV spectra, the λ values of the maximum and of the shoulder are indicated.

at *m/z* 1125.3 originated an intense signal at *m/z* 1107.6 (Figure 2S, Supplementary Material). These MS/MS spectra indicate that the parent ions did not easily fragmented by the collision energy of 30 ev used in this study, and this is probably due to the typical lantibiotic structure, where the presence of (Me)Lan bridges requires higher collision energy for generating fragments.

When these UV and MS data were matched with the information stored in databases ABL [20, 28] and Antibase, the compound eluting at 11.7 min present in the broth crude extract (and to a lesser extent in the mycelium) was identified as the A2 congener of microbisporicin, while the compound eluting at 12.2 from the mycelium extract (and to a lesser extent from the broth extract) was identified as the A1 congener of microbisporicin. It is important to note that A1 and A2 congeners of microbisporicin differ for the presence of dihydroxy- or hydroxyl-proline in the aminoacidic sequence, equivalent to a difference of one oxygen in the molecular formula, respectively, $C_{94}H_{127}ClN_{26}O_{27}S_5$ and $C_{94}H_{127}ClN_{26}O_{26}S_5$. Thus, the difference observed through LC/MS/MS between F31/11 active component eluting at 11.7 and F31/11 active component eluting at 12.2 min (Figure 2S in the Supplementary Material) could be explained by the presence of an additional oxygen on proline. Figure 3S in Supplementary Material confirms that when the A1 congener of microbisporicin was analyzed by LC/MS/MS in parallel with the compound eluting at 12.2 min, the two molecules originate the same fragmentation signals, reported in Figure 3S of the Supplementary Material. The identification of the two active components produced by F31/11 as the A1 and A2 congeners of microbisporicin was then further confirmed by LC-UV-MS analyses of F31/11 extracts in parallel with standards of actagardine, planosporicin, and microbisporicin (Table 3).

Microbisporicin is the most potent antibacterial among the known lantibiotics [8]; under the commercial name of NAI-107, it is currently in late pre-clinical phase for the treatment of multi-drug resistant Gram-positive pathogens [12, 13]. So far, two actinomycetes both belonging to the *Microbispora* genus have been reported to produce a different complex of microbisporicin congeners: *Microbispora* sp. 107981 mostly produces A1 and A2 congeners differing by the presence of dihydroxy- or hydroxyl-proline at position 14 in the 24 amino acid long scaffold [8]. Other minor congeners

Table 3: Retention time and typical UV and mass signals of actagardine and planosporicin and of major microbisporicin congeners in the LC-UV-MS system described in Section 2. Mass signals are reported in Dalton. λ_1 and λ_2 signals indicate, respectively, lambda (max) and lambda (shoulder).

ANTIBIOTIC	M	r.t. (min)	$[M + 2H]^{2+}$	$[M - 2H]^{2-}$	$[M + H]^{+}$	UV nm (λ_1 and λ_2)
Actagardine	1889	10.6	944.5	943.5	1890	227, 282
Microbisporicin A1	2246	12.2	1125.3	1123.4	2247	226, 267
Microbisporicin A2	2230	11.7	1117.2	1115.3	2231	225, 267
Microbisporicin 1768α	2214	12.8	1108.5	—	2215	223, 270
Microbisporicin 1768β	2180	9.6	1091	—	2181	223, 270
Planosporicin	2196	8.7	1099.7	1097.7	2197	225, 279, 288
F31/11 broth extract	2230	11.7	1117.2	1115.2	2231	225, 267
F31/11 mycelium extract	2246	12.2	1125.3	1123.4	2247	226, 267

produced by the same strain have been recently identified, carrying possible permutations on the tryptophan residue at position 4 (no modification or chlorination) and on the proline at position 14 (no modification or mono- or di-hydroxylation) [31]. *Microbispora corallina* NRRL 30420 produces mostly 1768β (no modification on proline at position 14) and 1768α (not chlorination on tryptophan at position 4 and no modification on proline at position 14) and lower amount of A1 and A2 [31–33]. We cannot exclude that other minor components could be produced by F31/11 strain, but the data reported in Table 3 indicate that, in the cultivation conditions so far used, it coproduces A2 and A1 congeners, preferentially accumulating A2 into the broth. We can add that the isotopic profile of the mass spectrum of F31/11 active peaks confirms the presence of chlorine in the molecule (data not shown).

3.4. Characterization of the F31/11 Producer Strain. Isolates belonging to the F.I.I.R.V. microbial collection were initially attributed to the *Actinoallomurus* genus mainly on the basis of their morphological and physiological features and by 16S rRNA gene sequencing [23, 24]. Typically, *Actinoallomurus* sp. F31/11 grows well at 30–37°C on ISP3 agar acidified to pH 5.5–6.0 with HCl. It forms typical chains of looped spores (Figure 2); the substrate mycelium is convolute and the mass colour of the substrate mycelium is cream. Good production of white-grey aerial mycelium was observed after 15 days of incubation. No soluble pigments are produced.

The taxonomical affiliation of strain F31/11 to the genus *Actinoallomurus* was confirmed by pairwise comparison of its almost complete 16S rRNA gene (1400 bp) with those of already described members of the *Actinoallomurus* genus (Figure 3) [23]. F31/11 16S rRNA sequence showed an identity of 99% with *Actinoallomurus yoronensis, Actinoallomurus fulvus, Actinoallomurus caesius*, and *Actinoallomurus amamiensis*. This identity value is indeed lower than 99.5%, which is considered the threshold for distinguishing different phylotypes; thus, F31/11 might be considered a novel species. The phylogenetic tree shown in Figure 3 clearly indicates that F31/11 with other *Actinoallomurus* spp. form a distinct clade within the *Thermomonosporaceae* family and that

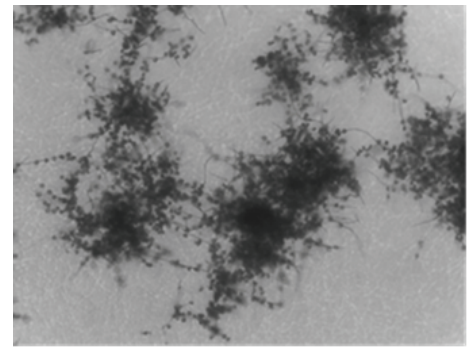

Figure 2: Morphology of F31/11 observed at the light microscope (model ULWD-CDPlan; Olympus, with 40x magnification).

F31/11 is quite distant from the microbisporicin producer *Microbispora corallina* (*Streptosporangiaceae* family) as well as from other lantibiotic producing actinomycetes such as *Planomonospora alba* (*Streptosporangiaceae* family) that produces planosporicin [7, 34] and from *Actinoplanes garbadinensis* and *Actinoplanes liguriensis* (*Micromonosporaceae* family) that produce actagardine [35].

4. Conclusions

As far as we know, this is the first report on a lantibiotic produced by an *Actinoallomurus* sp. and on a microbisporicin producer not belonging to the *Microbispora* genus. Unrelated compounds belonging to different chemical classes (benanomicin, coumermycin, N-butylbenzensulphonamide, and halogenated spirotetronates) have been recently discovered as products of *Actinoallomurus* spp. [24, 25], confirming that this novel genus represents a promising source for discovering novel bioactive metabolites when targeted with selective and efficient screening strategies. While most lantibiotics have been previously isolated and characterized from different genera of *Firmicutes*, recent investigations [7, 8, 18, 31–34] indicate that uncommon actinomycetes (non-streptomyces actinomycetes) can effectively contribute to the discovery of novel and useful lantibiotics. The case reported

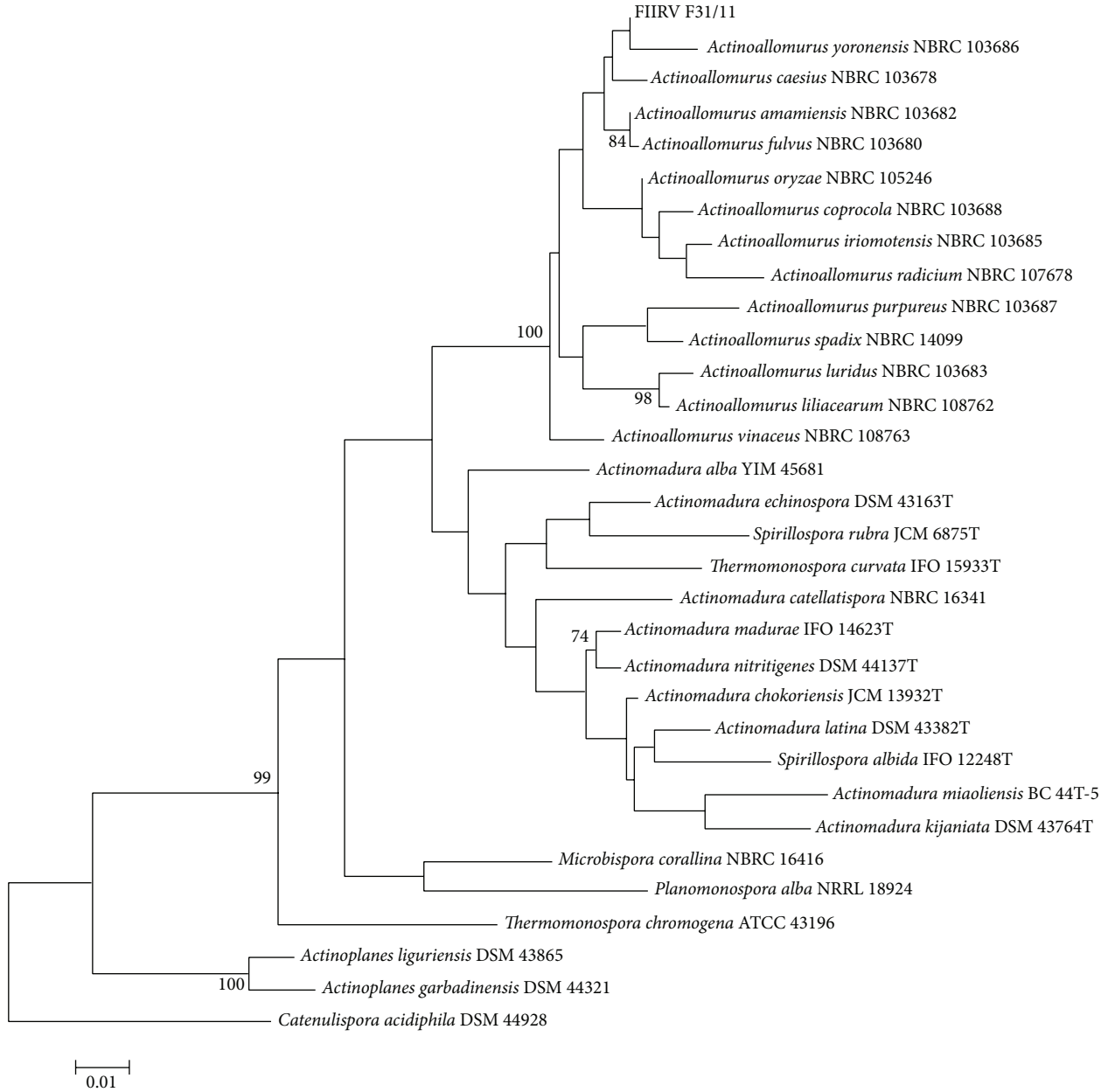

Figure 3: Phylogenetic tree derived from the 16S rRNA gene sequences of *Actinoallomurus* species and related actinomycetes belonging to the *Thermomonosporaceae* family. Sequences from actagardine, planosporicin, and microbisporicin actinomycete producers were also included. For the construction of the phylogenetic tree, selected sequences were aligned with Clustal-Omega (from the EMBL-EBI site) and analyzed with BioEdit [30]. Distance matrices were calculated with MEGA5.2, using the Maximum Likelihood method implemented in the program and the method of Jukes and Cantor. Trees were inferred using the Nearest-Neighbor-Interchange (NNI) heuristic method and making the initial tree with both Neighbour Joining and BioNJ, and selecting the superior tree (all methods are included in the MEGA package). All analyses were performed on a bootstrapped data set containing 500 replicates.

here suggests that same lantibiotic scaffolds may be produced by diverse families of actinomycetes. Thus, coupling an intelligent biological-activity guided screening with an early efficient dereplication approach avoid spending time in labour intensive procedure of purification and structural elucidation of already known metabolites. As recently reviewed in [36], implementing efficient, early LC-MS dereplication platform to identify known compounds in natural product databases containing their spectra, is nowadays considered a strategic step in natural product discovery. Further investigations will be devoted to understanding the potential of *Actinoallomurus* spp. as specialized metabolite producers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Improved Production of Sublancin 168 Biosynthesized by *Bacillus subtilis* 168 Using Chemometric Methodology and Statistical Experimental Designs

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Sublancin 168, as a distinct S-linked antimicrobial glycopeptide produced by *Bacillus subtilis* 168, is effective in killing specific microorganisms. However, the reported yield of sublancin 168 is at a low level of no more than 60 mg from 1 L fermentation culture of *B. subtilis* 168 by using the method in the literature. Thus optimization of fermentation condition for efficiently producing sublancin 168 is required. Here, Box-Behnken design was used to determine the optimal combination of three fermentation parameters, namely, corn powder, soybean meal, and temperature that were identified previously by Plackett-Burman design and the steepest ascent experiment. Subsequently, based on the response surface methodology, the quadratic regression model for optimally producing sublancin 168 was developed, and the optimal combination of culture parameters for maximum sublancin 168 production of 129.72 mg/L was determined as corn powder 28.49 g/L, soybean meal 22.99 g/L, and incubation temperature 30.8°C. The results showed that sublancin 168 production obtained experimentally was coincident with predicted value of 125.88 mg/L, and the developed model was proved to be adequate, and the aim of efficiently producing sublancin 168 was achieved.

1. Introduction

Sublancin 168 is a novel and distinct S-linked bacteriocin glycopeptide consisting of 37 amino acids and is produced by *Bacillus subtilis* 168 strains [1, 2]. Based on its potent antimicrobial activity in inhibiting special Gram-positive bacteria, including *B. megaterium*, *B. subtilis* 6633, the pathogenic microbes *Streptococcus pyogenes,* and *Staphylococcus aureus* [1], this antimicrobial peptide could be used in a wide range of commercial applications, such as agriculture, cosmetics, and pharmaceutical field. However, the host strain was cultured with a medium used for producing subtilin [1, 3], which contains (per liter) sucrose 20 g, citric acid 11.7 g, Na_2SO_4 4 g, $(NH_4)_2HPO_4$ 4.2 g, yeast extract 5 g, 100 mL of a salt mixture (KCl 7.62 g, $MgCl_2{\cdot}6H_2O$ 4.18 g, $MnCl_2{\cdot}4H_2O$ 0.543 g, $FeCl_3{\cdot}6H_2O$ 0.49 g, and $ZnCl_2$ 0.208 g in 1000 mL H_2O), and sufficient NH_4OH to bring the pH to 6.8–6.9. As a result of complex medium ingredients and nonspecialized medium for producing sublancin 168, some events of the pinkish-brown color, fruity odor, and pH value near 6 that accompanying with good sublancin 168 production did not always occur, whereupon the production of sublancin 168 was usually at a low level of no more than 60 mg from 1 L bacterial culture [1]. The low yield of sublancin 168 has constrained its commercial application, and the optimization of fermentation conditions is required to allow for efficient production of sublancin 168.

Recently, there has been an increasing interest in response surface methodology processes for improving productivities of natural bioactive agents [4, 5]. Several bioactive proteins, such as eicosapentaenoic acid [6], tostadin [7], and antimicrobial compounds [8, 9], produced by bacteria strains optimized through response surface methodology have been recorded. However, literature is lacking cultivation optimization of sublancin 168 produced by *B. subtilis* 168 using chemometric and statistical methodology.

The aim of this current work was to efficiently produce sublancin 168 via optimizing the variables of medium compositions and culture conditions by using statistical tools in shake-flasks. In the first step, Plackett-Burman design as an

effective technique was used to screen remarkable variables. Subsequently, the steepest ascent was utilized to approach the optimal region. At last, Box-Behnken design and response surface analysis were employed to ascertain the optimum levels of the factors which significantly effect sublancin 168 productions. In this study, the sublancin 168 production at a high level is achieved through adopting chemometric and statistical methodology.

2. Materials and Methods

2.1. Materials. Yeast extract and tryptone were purchased from Difco (Detroit, USA). Corn powder and soybean meal were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. (China) and Shandong Litong Biotechnology Co., Ltd. (China), respectively, and were passed through 60-mesh sieve. Other chemicals used were of chemical grade.

2.2. Bacteria Strains and Fermentation Condition. *B. subtilis* 168 (ATCC 27370), the producer of sublancin 168, was utilized in the current work [1]. The strains were maintained on Luria-Bertani medium (LB) agar slant with the following composition (g/L): tryptone 10.0, yeast extract 5.0, NaCl 5.0, and agar 18.0 with the pH value to 7.0. After culturing at 37°C for 28 hours, the slants were subcultured once a month and stored at 4°C.

Seed culture of *B. subtilis* 168 was prepared by culturing bacterial strains in a 250 mL flask containing 50 mL LB liquid medium at 37°C, 225 rpm for 12 hours. Subsequently, 1 mL prepared seed culture was inoculated into 250 mL flask containing 50 mL culture medium (g/L): peptone 10.0, starch 15.0, KH_2PO_4 4.0, and $(NH_4)_2SO_4$ 4.0, and the pH was adjusted to 7.0, and furtherly cultured for 48 hours. After fermentation, the culture supernatant was harvested by removing the cells and the debris through centrifugation 10,000 g for 5 min. Each test was repeated three times and the average of sublancin 168 concentration was taken as the response.

2.3. Screen of Carbon and Nitrogen Sources. The optimal nitrogen and carbon sources effecting sublancin 168 production were screened by one variable at a time (OVAT) approach. The evaluations of different simple and complex nitrogen (yeast extract, peptone, soybean meal, urea, and $(NH_4)_2SO_4$) and carbon sources (corn powder, glycerol, sucrose, lactose, starch, maltose, and glucose) on sublancin 168 production were performed one by one (Table 1). The above different nitrogen sources (5 g/L) and carbon sources (10 g/L) instead of peptone and starch were taken into the culture procedure as described above.

2.4. Plackett-Burman Design. The Plackett-Burman design is a powerful tool for rapidly screening and determining the important variables that has significant influence on the production response. This method was very useful for picking the most important factors from a long list of candidate factors [10]. In this work, different cultivation parameters (inoculum size, initial pH, incubation temperature, and incubation time) and medium components (peptone, soybean meal, starch, corn powder, KH_2PO_4, and $(NH_4)_2SO_4$) were evaluated utilizing Plackett-Burman design to identify the important factors influencing sublancin 168 production greatly. Each factor at two levels was examined based on Plackett-Burman factorial design: −1 and +1 for low and high level, respectively [11]. On the preliminary study, it was found that the optimal temperature for producing sublancin 168 by *B. subtilis* 168 was at 32°C. Therefore, in Plackett-Burman experiment, the culture temperature test level was set between 30°C and 34°C. Table 2 illustrates the factors under investigation and the levels of each factor setting in the experimental design. Response values were determined based on sublancin 168 productions.

Table 1: Effects of different carbon sources and nitrogen sources on the yield of sublancin 168.

Carbon sources		Nitrogen sources	
Sources	Yield (mg/L)	Sources	Yield (mg/L)
Corn powder	67.66 ± 3.56	Yeast extract	28.89 ± 4.72
Glycerol	28.96 ± 4.39	Peptone	50.11 ± 4.56
Sucrose	30.55 ± 4.90	Soybean meal	58.40 ± 5.33
Lactose	29.61 ± 5.03	Urea	8.72 ± 3.18
Starch	50.65 ± 4.85	$(NH_4)_2SO_4$	32.93 ± 2.10
Maltose	28.26 ± 5.10		
Glucose	21.40 ± 5.49		

Each experiment was repeated three times, and all of the data were expressed as means ± standard deviations.

The Plackett-Burman design was established by SAS software package (version 9.1.3, SAS Institute Inc., Cary, NC, USA) in terms of the following first-order model:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i, \quad (1)$$

where Y refers to the response (i.e., sublancin 168 production) and β_0, β_i, X_i, and k represent the constant, the linear coefficient, the level of the independent variables, and the number of involved variables, respectively.

In addition to the variables of real interest, the Plackett-Burman design considers insignificant dummy variables, which are introduced to evaluate the experimental error and the variance of the first-order model. In this work, 10 variables were checked in 20 trials (Table 3). Every trial was performed three times, and the average sublancin 168 production was applied as the response variable. Regression analysis determined the variables that had a significant effect ($P < 0.05$) on sublancin 168 production, and these variables were subsequently evaluated in further optimization experiments.

2.5. Steepest Ascent Method. In general, some variations of the optimum culture condition for the system exist between the actual optimum and the initial estimate. In such case, the single steepest ascent experiment was performed to optimize the variables influenced sublancin 168 production significantly [12].

Table 2: Variables and test levels for Plackett-Burman experiment.

Number	Variables	Code levels		Estimate	t-value	P value	Significance
		−1	1				
X_1	Peptone (g/L)	8	12	2.33	1.01	0.3851	
X_2	Corn powder (g/L)	20	30	27.20	11.83	0.0013	*
X_3	Starch (g/L)	10	20	3.19	1.39	0.2594	
X_4	Soybean meal (g/L)	24	36	21.09	9.17	0.0027	*
X_5	KH_2PO_4 (g/L)	3	6	3.21	1.39	0.2575	
X_6	$(NH_4)_2SO_4$ (g/L)	3	6	−0.96	−0.42	0.7038	
X_7	Incubation temperature (°C)	28	34	11.11	4.83	0.0169	*
X_8	Initial pH	6.5	8.5	−1.91	−0.83	0.0466	
X_9	Incubation time (h)	28	40	0.38	0.17	0.8786	
X_{10}	Inoculum size (%)	1	3	−2.34	−1.01	0.3834	

* indicates model terms are significant.

Table 3: Experimental design and results of the Plackett-Burman design.

Trials	Variable levels										Yield (mg/L)	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	Observed	Predicted
1	1	−1	1	1	−1	−1	−1	−1	1	−1	88.38 ± 3.84	90.91
2	1	1	−1	1	1	−1	−1	−1	−1	1	117.96 ± 2.37	115.41
3	−1	1	1	−1	1	1	−1	−1	−1	−1	97.42 ± 6.78	96.55
4	−1	−1	1	1	−1	1	1	−1	−1	−1	101.79 ± 3.41	98.33
5	1	−1	−1	1	1	−1	1	1	−1	−1	102.43 ± 3.74	99.73
6	1	1	−1	−1	1	1	−1	1	1	−1	92.45 ± 3.96	94.17
7	1	1	1	−1	−1	1	1	−1	1	1	107.47 ± 9.89	104.83
8	1	1	1	1	−1	−1	1	1	−1	1	121.38 ± 2.26	124.57
9	−1	1	1	1	1	−1	−1	1	1	−1	119.62 ± 2.27	117.05
10	1	−1	1	1	1	1	−1	−1	1	1	88.76 ± 5.85	90.81
11	−1	1	−1	1	1	1	1	−1	−1	1	119.63 ± 5.56	123.21
12	1	−1	1	−1	1	1	1	1	−1	−1	79.81 ± 4.77	80.87
13	−1	1	−1	1	−1	1	1	1	1	−1	118.85 ± 5.84	120.81
14	−1	−1	1	−1	1	−1	1	1	1	1	76.33 ± 3.15	77.53
15	−1	−1	−1	1	−1	1	−1	1	1	1	82.21 ± 2.79	80.17
16	−1	−1	−1	−1	1	−1	1	−1	1	1	77.19 ± 4.41	76.27
17	1	−1	−1	−1	−1	1	−1	1	−1	1	62.37 ± 5.76	61.05
18	1	1	−1	−1	−1	−1	1	−1	1	−1	106.22 ± 7.25	104.95
19	−1	1	1	−1	−1	−1	−1	1	−1	1	90.56 ± 2.95	90.05
20	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	60.31 ± 3.20	63.93

Each experiment was repeated three times, and all of the data were expressed as means ± standard deviations. $X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9$, and X_{10} represent peptone (g/L), corn powder (g/L), starch (g/L), soybean meal (g/L), KH_2PO_4 (g/L), $(NH_4)_2SO_4$ (g/L), incubation temperature (°C), initial pH, incubation time (h), and inoculum size (%).

2.6. Response Surface Methodology. Through the Plackett-Burman design experiment, the significant variables were selected as follows: soybean meal, corn powder, and incubation temperature. After that, the Box-Behnken design, a type of response surface methodology, was used to determine the optimum level of these selected variables for producing sublancin 168 as highly as possible. With the help of the statistical software package "Design Expert 8.0.5b" (Shanghai TechMax Co., Ltd., Shanghai, China), the experimental design was analyzed and 15 experiments in all were formulated. The central values of every variable were coded 0. The maximum and minimum ranges of the variables were set up, and the whole experiment program in terms of their coded and actual values is shown in Table 5. In all trials the response values (Y) were the average of three replicates.

2.7. Batch Fermentation in a 5 L Bioreactor. To investigate the behaviour of sublancin 168 accumulation, batch fermentations were conducted in a 5 L bioreactor (NBS Co., USA). The prepared seed culture was inoculated (2%, v/v) into the optimal medium with an initial pH 7.0. According to the preexperiment results (data not shown), the bioreactor was operated with optimized temperature, airflow at 1.5 vvm, and stirring at 500 rpm, and the pH was uncontrolled during fermentation.

Table 4: Experimental design and corresponding response of steepest ascent.

Experiment number	Corn powder (g/L)	Soybean meal (g/L)	Incubation temperature (°C)	Yield (mg/L)
0	12	8	25	72.7 ± 2.97
0 + 1Δ	16	12	27	80.3 ± 4.11
0 + 2Δ	20	16	29	89.5 ± 2.42
0 + 3Δ	24	20	31	117.5 ± 3.58
0 + 4Δ	28	24	33	122.6 ± 1.64
0 + 5Δ	32	28	35	109.0 ± 4.17

Each experiment was repeated three times, and all of the data were expressed as means ± standard deviations.

Table 5: Experimental design and results of Box-Behnken optimization experiment.

Trials	X_1	X_2	X_3	Yield (mg/L)	
				Observed	Predicted
1	22.00	24.00	36.00	73.66 ± 1.62	73.43
2	34.00	28.00	32.00	86.35 ± 2.78	86.78
3	28.00	28.00	28.00	77.37 ± 1.83	77.57
4	28.00	24.00	32.00	124.39 ± 1.92	124.15
5	22.00	28.00	32.00	71.97 ± 1.26	71.81
6	34.00	24.00	28.00	94.52 ± 3.73	94.75
7	28.00	28.00	36.00	92.97 ± 5.06	93.37
8	28.00	20.00	28.00	115.92 ± 2.90	115.53
9	22.00	20.00	32.00	86.35 ± 2.72	85.93
10	28.00	24.00	32.00	124.22 ± 1.24	124.15
11	28.00	24.00	32.00	123.85 ± 1.63	124.15
12	34.00	20.00	32.00	92.67 ± 3.56	93.37
13	34.00	24.00	36.00	90.80 ± 2.90	90.82
14	22.00	24.00	28.00	91.98 ± 3.72	91.95
15	28.00	20.00	36.00	77.48 ± 2.35	77.28

Each experiment was repeated three times, and all of the data were expressed as means ± standard deviations. X_1, X_2, and X_3 represent corn powder (g/L), soybean meal, and incubation temperature (°C), respectively.

2.8. Quantitative Determination of Sublancin 168 Content. Isolation and purification of sublancin 168 were carried out as previously described [1], with slight modification. The collected supernatant was made in 1 M NaCl and subjected to a hydrophobic interaction chromatography of 25 mL Toyopearl Butyl-650 column (Tosoh, Tokyo, Japan), and then a solution of 50 mM NaAc, pH 4.0, was used to wash down the sublancin. Subsequently the elution was made in 0.1 trifluoroacetic acid (TFA) and subjected to a semipreparative Zorbax 300SB-C8 column (250 × 9.4 mm, 5 μm particle size, 300 Å pore size) (Agilent, Englewood, CO) with a linear 0–60% acetonitrile gradient at a flow rate of 1.0 mL/min. The active fractions were collected and applied to an analytical Zorbax 300SB-C8 column (150 × 4.6 mm, 5 μm particle size, 300 Å pore size) (Agilent, Englewood, CO) with the same conditions as the first step. The absorbances at 214 nm, 254 nm, and 280 nm were monitored. The concentration of purified sublancin 168 was determined by UV spectrophotometry [13, 14]. Using purified sublancin as standard sample, the fermentation broths were applied to analytical Zorbax 300SB-C8 column to determine sublancin 168 concentrations with the method used in purification of sublancin 168.

2.9. Statistics. During this study, each experiment was repeated three times, and all of the data were expressed as means ± standard deviations.

3. Results and Discussion

3.1. Screening Optimal Carbon Sources and Nitrogen Sources. According to the fermentation result (data not shown) obtained by using the method reported in the literatures [1, 3], there is a no more than 60 mg sublancin 168 from one liter bacterial culture. Thus, a new fermentation method with some different media and culture conditions is required to efficiently produce sublancin 168. As illustrated in Table 1, among the evaluations with different nitrogen sources, soybean meal showed an outstanding effect on the sublancin 168 production of 58.40 mg/L, followed by peptone of 50.11 mg/L. Urea had played an insignificant role on this peptide production. Among the different tested carbon sources, corn powder had a prominent effect on the sublancin 168 production of 67.66 mg/L, followed by starch of 50.65 mg/L, and glycerol had a sight effect on the yield of sublancin 168. Corn powder and soybean meal play an important role

Table 6: Analysis of variances of the quadratic polynomial model.

Source	SS	DF	MS	F-value	$P > F$
Model	4776.00	9	530.67	2507.25	<0.0001
Lack of fit	0.90	3	0.30	3.92	0.2100
Pure error	0.15	2	0.08		
Total	4777.05	14			

$R^2 = 0.9998$, $R^2_{\text{Adj}} = 0.9993$, and $R^2_{\text{Pre}} = 0.9998$. SS: sum of squares; DF: degrees of freedom; MS: mean square.

in production improvement of interest products, such as *Acinetobacter* sp. DNS_{32} strain [15], *B. subtilis* WHK-Z12 spore [16], β-glucanase from *B. subtilis* ZJF-1A5 [17], and alkaline protease from *Bacillus* sp. RKY3 [18]. In this study, the results of screening optimal carbon sources and nitrogen sources suggested that corn powder and soybean meal were also important for *B. subtilis* 168 strains to efficiently produce sublancin 168.

3.2. Plackett-Burman Design. The Plackett-Burman design was employed to evaluate the relative importance of cultivation parameters and different medium components (Figure 1). The main effect of each variable upon sublancin 168 production was evaluated as the difference made between both averages of measurements at the high level (+1) and at the low level (−1) correspondingly (Table 2). The data in Table 3 showed a wide variation from 60.31 mg/L to 121.38 mg/L. The results suggested that higher productivity of sublancin 168 was achieved from medium optimization. The significant variables affecting sublancin 168 productivity are soybean meal, corn powder, and incubation temperature from the calculation of t-values and P values (Table 2 and Figure 1). The incubation time from 28 h to 40 h and inoculum size from 1% to 3% play an insignificant role on sublancin 168 production.

Based on (1) and analyzed by using Minitab, a first-order model was fitted to the results obtained from the twenty experiments as the following equation:

$$\begin{aligned} Y\ (\text{mg/L}) = {} & 95.56 + 1.17X_1 + 13.60X_2 + 1.60X_3 + 10.54X_4 \\ & + 1.60X_5 - 0.48X_6 + 5.55X_7 - 0.96X_8 \\ & + 0.19X_9 - 1.17X_{10}, \end{aligned} \tag{2}$$

where Y is the sublancin 168 production and X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} are the coded values of peptone, corn powder, starch, soybean meal, KH_2PO_4, $(NH_4)_2SO_4$, incubation temperature, initial pH, incubation time, and inoculum size, respectively. The goodness of the regression model was determined by the coefficient of determination R^2 whose value is 96.76% and suggests that only 3.24% of the total variation could not be explained by the model. Hence it was reasonable to take the regression model to analyse the tread in the response.

3.3. Steepest Ascent. Even though Plackett-Burman design allows for the rapid selection of the significant variables affecting productivity of sublancin 168, the optimum levels of the variables cannot be predicted by this method. The method of steepest ascent is a procedure for moving sequentially along the path of steepest ascent and in the direction of the maximum increase in the response. In order to move the variables rapidly to the general vicinity of the optimum levels, the path of steepest ascent was used to find the proper direction to change the variables by increasing the incubation temperature and the concentration of soybean meal and corn powder to improve the production of sublancin 168. The results showed that the sublancin 168 production reached a yield plateau during the fifth step (Table 4). Thus, these three variables were selected for further optimization.

As illustrated in Table 4, the sublancin 168 production did not further increase with the increase of concentrations of corn powder and soybean meal and the increase of temperature. For corn powder and soybean meal, the yield of sublancin 168 decreased from 122.6 mg/L to 109.0 mg/L when the concentrations of corn powder and soybean meal increased from 28 g/L to 32 g/L and from 24 g/L to 28 g/L, respectively. The increase temperature from 33°C to 35°C may make the cells more difficult to biosynthesize sublancin 168; therefore, the sublancin production was not improved.

3.4. Box-Behnken Design. To determine the optimum levels of these important independent variables (soybean meal, corn powder, and incubation temperature) according to the above results, a 3-factor Box-Behnken design with 3 levels involving 3 replicates at center point was introduced to fit a second-order response surface. Table 5 shows the design matrix and the corresponding experimental data. The quadratic regression equations were obtained according to sublancin 168 production after the above results were analysed through standard analysis of variance (ANOVA). With the data of designed experiments, the polynomial regression model (in coded value) for sublancin 168 yield Y was regressed only with respect to the significant factors and was shown as follows:

$$\begin{aligned} Y = {} & 124.15 + 5.07X_1 - 5.51X_2 - 5.63X_3 + 2.06X_1X_2 \\ & + 3.65X_1X_3 + 13.47X_2X_3 - 21.51X_1^2 - 18.35X_2^2 \\ & - 14.91X_3^2, \end{aligned} \tag{3}$$

where Y predicates the sublancin 168 production, X_1 is corn powder, X_2 is soybean meal, and X_3 is incubation temperature.

Based on F-test and ANOVA using the SAS software package, the statistical significance of (3) was evaluated. As shown in Table 6, F-value of the model is 2507.25, and F-value

Table 7: Results of regression analysis of the second-order polynomial model.

Factor	Coefficient estimate	Standard error	F-value	$P > F$
Intercept	124.15	0.27	2507.25	<0.0001
X_1	5.05	0.16	963.01	<0.0001
X_2	−5.47	0.16	1130.53	<0.0001
X_3	−5.61	0.16	1189.84	<0.0001
X_1X_2	2.02	0.23	76.82	0.0003
X_1X_3	3.65	0.23	251.72	<0.0001
X_2X_3	13.51	0.23	3449.98	<0.0001
X_1^2	−21.51	0.24	8068.33	<0.0001
X_2^2	−18.31	0.24	5847.41	<0.0001
X_3^2	−14.91	0.24	3877.87	<0.0001

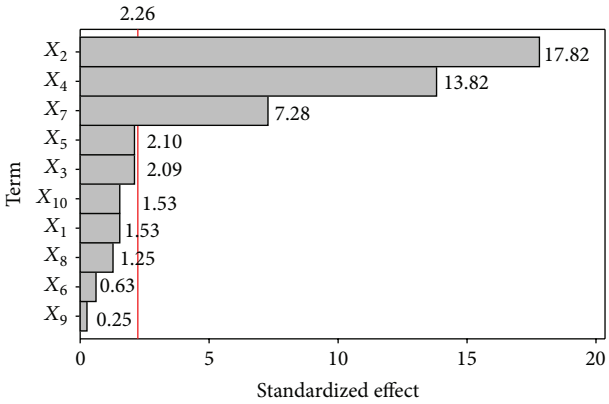

Figure 1: Pareto chart of standardized effects on sublancin 168 production. X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} are the coded values of peptone, corn powder, starch, soybean meal, KH_2PO_4, $(NH_4)_2SO_4$, incubation temperature, initial pH, incubation time, and inoculum size, respectively. The chart has a vertical line (i.e., standardized effect = 1.886) at the critical t-value for α of 0.20. The bars are shown in order of the size of the effects, and the standardized effect of every term was displayed on the top of its corresponding bar.

for lack of fit is 3.92. The high F-value and nonsignificant lack of fit indicate that the model (3) is a good fit. This result indicates that the model used to fit response variable is significant ($P < 0.0001$) and adequate to represent the relationship between the responses and the independent variables. And the ANOVA (F-test) for this work is shown in Table 7. The value of determination coefficient R^2 is 0.9996, which means that we are able to explain 99.98% of results for sublancin 168 production using the calculated model. This result indicates that the model used to fit response variable is significant ($P < 0.0001$) and adequate to represent the relationship between the responses and the independent variables. Meantime, the significance of the model was satisfactorily confirmed by the adjusted determination coefficient ($R^2_{\text{Adj}} = 0.9993$) and predicated determination coefficient ($R^2_{\text{pre}} = 0.9998$). Thus using this model to estimate the response trends is considered to be reasonable.

The model coefficient calculated from the regression analysis for each significant variable is shown in Table 7. Table 7 shows that the regression coefficients of individual linear, quadratic terms, and two cross products are statistically significant at 95% confidence level.

Three-dimensional (3D) response surface plots (Figure 2(a)) and two-dimensional (2D) contour plots (Figure 2(b)) are the graphical representations of the quadratic polynomial regression equation and usually illustrate the relationships between the experimental levels of each variable and corresponding response. The sublancin 168 production is shown in Figure 2 by 3D response surface plots and their respective 2D contour plots. Each figure reveals the interaction of two variables meanwhile the other is kept at zero level. In the 3D response surface plots and 2D contour plots as shown, the interaction exists within every pair of selected three factors and the effects are significant.

The model reveals that the corn powder concentration (X_1), soybean meal concentration (X_2), and temperature (X_3) had a significant effect ($P < 0.0001$) on the sublancin 168 production (Y). Positive coefficient of X_1 indicated a linear effect to increase, and negative coefficient of X_2 and X_3 suggested a linear effect to decrease. Meanwhile, quadratic term X_1^2, X_2^2, and X_3^2 had the negative effect. The graphs (Figure 2) illustrate the changes in the parameter modelled as the two factors move along those levels, while the other factor held constant at the central point. According to (3), it was predicted that a maximum sublancin 168 production of 125.88 mg/L appeared at 22.99 g/L soybean meal and 28.49 g/L corn powder, while temperature was held at 30.8°C.

3.5. Model Verification in Shake-Flask. The availability of the regression model of the sublancin 168 production using the calculated optimal medium compositions and culture condition, namely, 22.99 g/L soybean meal, 28.49 g/L corn powder, and temperature at 30.8°C, was validated with triplicate experiments. The mean maximal value of sublancin 168 production was 129.72 mg/L, which agreed with the predicted value (125.88 mg/L) well. As a result, the model was considered to be accurate and reliable for predicting the sublancin 168 production by *B. subtilis* 168. However, there is a certain gap between the yield and the theoretical value, which may be caused by some factors other than the medium that affect the yield of sublancin 168 but not investigated in this work. In this study, the yield of sublancin 168 by *B. subtilis* 168 was improved from low level of no more than 60 mg/L up to 129.72 mg/L in optimized medium.

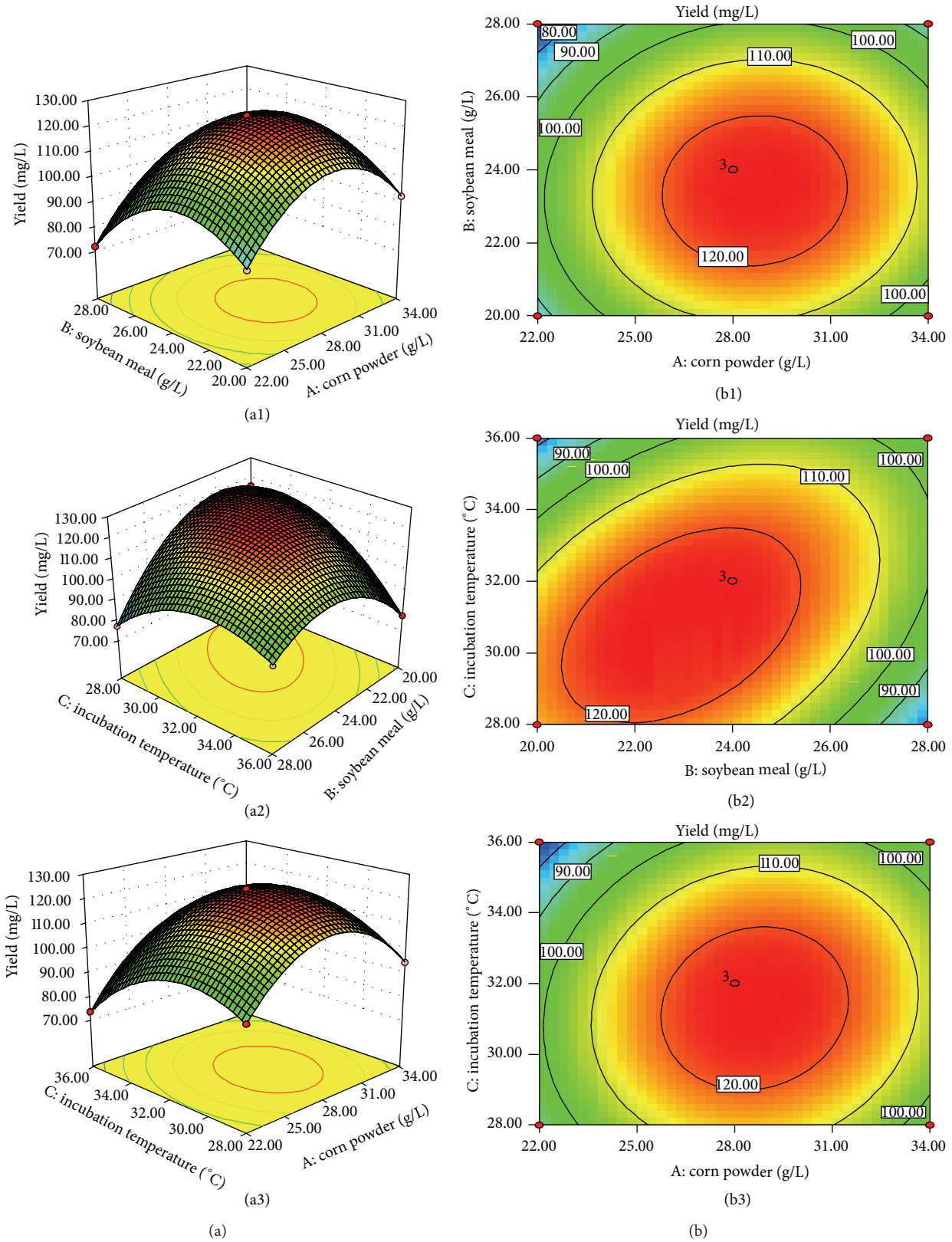

Figure 2: 3D response surface curves (a) and 2D contour plots (b) predicting for sublancin 168 production by *Bacillus subtilis* 168 through optimization of variables. The interaction between (a1, b1) soybean meal and corn powder, (a2, b2) incubation temperature, and soybean meal and (a3, b3) incubation temperature and corn powder.

3.6. Validation of the Model in Bioreactor. Using the optimal medium and temperature, sublancin 168 reached repeatable yield of 135.4 mg/L in bioreactor batch fermentation after about 48 h of cultivation. Although the temperature and medium components were coincident in flask and bioreactor fermentations, the yield of sublancin 168 in bioreactor fermentation (135.4 mg/L) was higher than that in the shake-flask culture (129.72 mg/L), probably mainly due to the differences of aeration conditions. Even so, the working conditions of the bioreactor require further optimizations in future experiments to furtherly improve sublancin yield.

3.7. Influence of Corn Powder and Soybean Meal on Sublancin 168 Production. In *B. subtilis* 168, there are six proteins (SunI, SunA, SunT, BdbA, SunS, and BdbB) [2] taking part in biosynthesizing mature sublancin 168, and the biosynthesis of sublancin 168 is controlled under a complex regulatory network that involves a minimum of five transcriptional regulators, including Abh, AbrB, Rok, YvrG, and YvrH [19–21]. Corn powder and soybean meal are commonly substrates used by bacteria to produce enzymes and other secondary metabolites through fermentation. *B. subtilis* 168 possesses an ATP-dependent protein kinase which can be activated by several metabolites (fructose 1,6-diphosphate, gluconate-6-P, and 2-phosphoglycerate) when growing in the presence of corn powder. The activated protein kinase phosphorylates a seryl residue (ser-46) of HPr, a small phosphocarrier protein [22]. HPr probably have a direct or indirect control function on the complex regulatory network that regulates the biosynthesis of sublancin 168. Soybean meal is a highly concentrated source of protein [23] and can provide an excellent profile of amino acids for producing sublancin 168.

4. Conclusions

As a summary, response surface methodology combined with Plackett-Burman design and steepest ascent enabled us to optimize the sublancin 168 yield produced by *B. subtilis* 168. The optimal combinations of culture parameters for maximum production of sublancin 168 were determined as corn powder 28.49 g/L, soybean meal 22.99 g/L, and incubation temperature 30.8°C, and the maximum production of sublancin 168 was significantly improved from no more than 60 mg/L before optimization up to 129.72 mg/L. To our knowledge, this is first report of statistical optimization for sublancin 168 production, which would provide some important parameters for large scale fermentation of this agent.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Shengyue Ji and Weili Li contributed equally to this work.

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Review Article

Antibacterial Discovery and Development: From Gene to Product and Back

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Concern over the reports of antibiotic-resistant bacterial infections in hospitals and in the community has been publicized in the media, accompanied by comments on the risk that we may soon run out of antibiotics as a way to control infectious disease. Infections caused by *Enterococcus faecium, Staphylococcus aureus, Klebsiella* species, *Clostridium difficile, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli*, and other *Enterobacteriaceae* species represent a major public health burden. Despite the pharmaceutical sector's lack of interest in the topic in the last decade, microbial natural products continue to represent one of the most interesting sources for discovering and developing novel antibacterials. Research in microbial natural product screening and development is currently benefiting from progress that has been made in other related fields (microbial ecology, analytical chemistry, genomics, molecular biology, and synthetic biology). In this paper, we review how novel and classical approaches can be integrated in the current processes for microbial product screening, fermentation, and strain improvement.

1. Introduction

Antibacterial therapy has saved millions of lives and considerably reduced the rate of premature death from bacterial infections. These achievements led to the assumption that pathogenic bacteria and the high mortality due to infectious diseases would be a thing of the past. Unfortunately, soon after the introduction of antibiotics, reports concerning the emergence of resistance started to accumulate. Antibiotic resistance mechanisms, which appear *de novo* or are transmitted among bacteria, have been well studied and described in many reviews. These include detoxification of antibiotic molecules and mutations in the designated target or, as described recently, are mediated by population-level resistance mechanisms [1]. It is now apparent that interspecies and intraspecies horizontal gene transfer of both Gram-negative and Gram-positive bacteria represent the dominant process by which bacteria become multiresistant. The selective pressure of antimicrobial use in hospitals, in communities, and in agriculture comprises the engine driving this process. Nowadays we are aware that bacterial resistance to all currently used antibiotics has emerged for both Gram-positive and Gram-negative bacteria. This threatening situation urgently calls for a concerted international effort among governments, the pharmaceutical industry, biotechnology companies, and the academic world to react and support the development of new antibacterial agents. One example of such initiative effort is the Infectious Diseases Society of America (IDSA) call to develop 10 new systemic antibacterial drugs by 2020 [2] by targeting drug development against both Gram-positive and Gram-negative bacteria. Unless serious action is taken, the acute and dangerous situation that exists today may send

us back to the preantibiotic era, when there was no cure for bacterial infections. If this happens, the prophecy of Louis Pasteur will be fulfilled and "microbes will have the last word."

2. Medical Needs for Novel Antibacterials

Multidrug-resistant bacterial infections represent a major public health burden, not only in terms of morbidity and mortality, but also in increased expenses for managing patients and implementing extensive infection control measures. Mortality due to multidrug-resistant bacterial infections is high. In 2002 it was reported that 1.7 million healthcare-associated infections occur each year in American hospitals and were associated with about 99,000 deaths [3]. This represents a huge increase from a previous estimation, which reported that in 1992 about 13,300 people died from hospital-acquired infection [4]. It is estimated that in the EU, Iceland, and Norway about 37,000 patients die as a direct result of a hospital-acquired infection each year; an additional 111,000 die as an indirect result of hospital-acquired infection [5]; and about 25,000 patients die from a multidrug-resistant bacterial infection.

Presently, the most frequent multidrug resistance (MDR) bacteria are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. which therefore were termed "ESKAPE" after initially being reported [6], with several reports adding *Clostridium difficile* or other *Enterobacteriaceae* [7]. Gram-positive pathogens, such as *Staphylococcus, Streptococcus, Enterococcus*, and *Clostridium*, account for a large proportion of serious infections worldwide. An increasing percentage of such Gram-positive isolates exhibit reduced susceptibility to first-line therapies [8–10], resulting in poor clinical outcomes in both community and hospital settings [10–13]; this has a significant impact on overall healthcare utilization and costs [10, 11]. *Staphylococcus aureus* and *Enterococcus* spp. were found to be among the most commonly isolated pathogens in the hospital environment, and being frequently resistant to multiple drugs complicates therapy. The representative hospital "superbugs," methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE), frequently attract mass-media attention and, in many countries, pressure is increasing to reduce MRSA and VRE infection rates. Resistance to anti-MRSA and anti-VRE drugs is uncommon; however, infections by MRSA strains resistant to glycopeptides, daptomycin, or linezolid (common anti-MRSA drugs) and by VRE strains resistant to daptomycin or linezolid (common anti-VRE drugs) are increasingly being reported, including reports of transferable resistance mechanism to these drugs among staphylococci and enterococci. In addition, reports regarding the emergence and spread of virulent clones of MRSA and *Clostridium difficile* in the community and in hospitals, respectively, have been published often. Moreover, multidrug-resistant *Streptococcus pneumonia* clones are currently considered major community pathogens in many parts of the world, although they are being challenged by new conjugate vaccines.

Although the prevalence of Gram-negative bacteria is currently somewhat lower than that of Gram-positive bacteria, it is well recognized that Gram-negative MDR infections are emerging as a threat to hospitalized patients with a significant impact on length of hospitalization, mortality, and cost [14, 15]. These include multiresistant nonfermenters, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, or multiresistant, extended-spectrum β-lactamase-producing *Enterobacteriaceae* and more recently carbapenem-resistant *Enterobacteriaceae* (CRE) of different types. Emerging resistance is due to the spread of the *Klebsiella pneumoniae* carbapenemase (KPC) and to the novel New Delhi metallo-β-lactamase (NDM-1). The rising crisis of multidrug-resistant Gram-negative bacteria has prompted the use of salvage therapy with colistin, an older polymyxin known to be neurotoxic and nephrotoxic [16, 17]. However, there are already reports describing isolates of several Gram-negative bacteria that are resistant to all available antibiotics, including polymyxins [18, 19].

3. Natural Product Discovery: The Screening Ingredients to Exploit Microbial Diversity

Despite the pharmaceutical sector's lack of interest in addressing the topic in the last decade, microbial products continue to represent one of the most interesting sources for the discovery of novel antibacterials today and research in the field is currently benefiting from progress that has been made in other related fields (microbial ecology, metagenomics, metabolomics, or synthetic biology), fields which have provided a deeper understanding of the microbiome and thus the development of new tools to foster the discovery of novel compounds. Among living organisms, microorganisms (actinobacteria, cyanobacteria, myxobacteria, and fungi) represent one of the most prolific sources for the production of antibiotics. For decades, exploitation of their specialized (commonly termed secondary) metabolism has guaranteed the discovery of novel antibiotics and other compounds with unprecedented chemical characteristics and biological properties that do not exist in the screening libraries of synthetic compounds [20, 21]. In this section, we examine the current trends in microbial product screening for discovering novel antibiotics. A flow diagram showing the overall screening operation is reported in Figure 1.

3.1. Microbial Product Libraries. Microbial natural product libraries rely on the quality and diversity of novel microbial strains and the approaches used to exploit their metabolic diversity. Access to microbial diversity traditionally focused on intensive sampling and isolation using general methods from a wide range of geographical locations and habitats, with recurrent isolation and screening of the predominant species and a low probability of isolating novel compounds. Although estimates for the potential production of unknown novel molecules by *Streptomyces* spp. [22] were high, the reality is that species spread widely in different environments produce the same well-known and structurally related antibacterial molecules. Current approaches oriented to

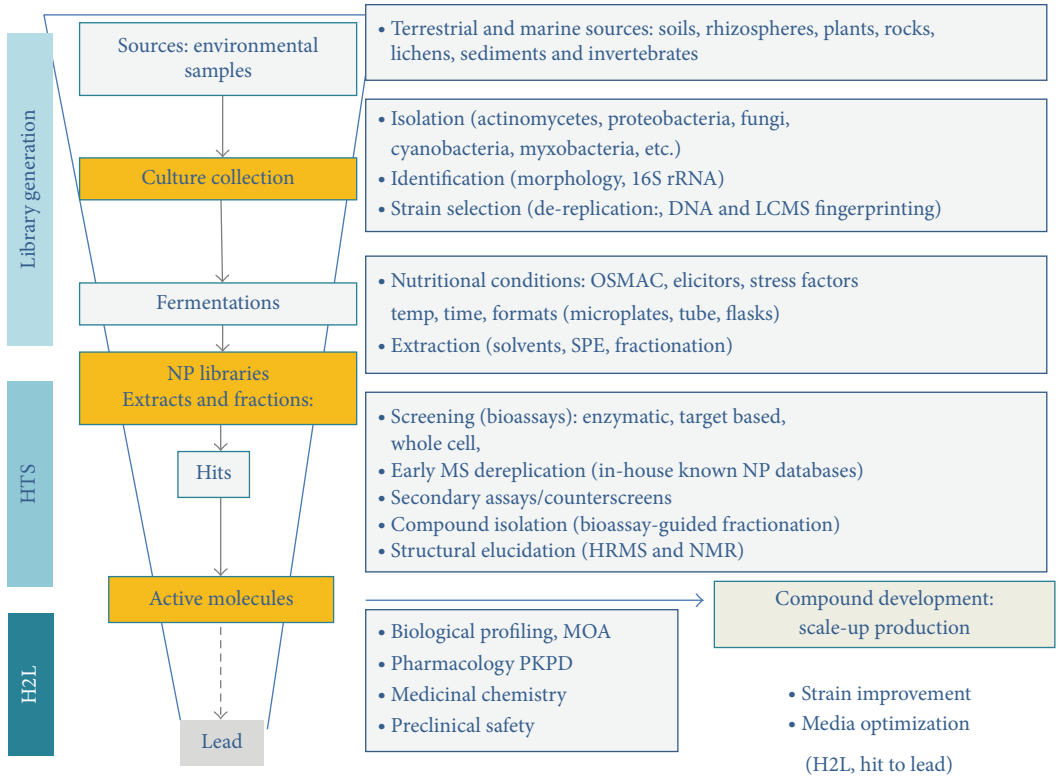

Figure 1: Early stages of antibiotic discovery from microbial product libraries.

discovering novel molecules mostly aim to target specific and minor microbial communities in unique or underexplored environments, including specific terrestrial niches, plant host-microbe associations, and marine environments. Environmental conditions comprise strong selecting factors and the distribution of some microbial species, even in highly occurring taxa, presents biogeographic patterns determined by microenvironmental conditions that can be translated into novel compounds. Many research groups have recently emphasized the exploration of untapped microbial communities that are associated with rhizospheres, plant endophytes, lichens, endolithic microbial communities, insect parasites, and endosymbionts and marine sediments and invertebrates. These approaches have favored the isolation of novel microbial communities potentially producing novel chemical scaffolds [23–27]. The search for novel sources has been combined with the use of novel isolation methods targeting the cultivation of species underrepresented or previously not cultivated under laboratory conditions [28–32]. Most of these methods are focused on selective isolation of the members of minor occurring taxa by using poor nutritional media devoid of carbon sources, subinhibitory concentrations of antibiotics that might favor the development of slow-growing representatives of these microbial communities after weeks of incubation, alternative gelling substrates to agar shown to prevent the growth of some microbial groups in laboratory conditions, *in situ* incubation chambers, or isolating endophytes that germinate directly on the substrate using humid chambers or by surface sterilization.

3.2. Tools for Strain Selection. Strain selection criteria are essential for building a strain collection and ensure the uniqueness of the isolates and that the widest microbial diversity is represented. Phenetic and molecular tools that can be applied hierarchically on the large numbers of isolates normally recovered from environmental samples have been intensively developed. These can include a simple morphological characterization of the growth and sporulating characteristics of actinomycetes and filamentous fungi at the macroscopic and microscopic levels, allowing preliminary assignment to a taxonomic group that can be complemented with the ribosomal gene sequencing of isolates in a large proportion of the cases. Partial ribosomal rDNA sequencing is frequently used to confirm the taxonomic affiliation and to assess in molecular data the microbial diversity and individual phylogenetic relationships within strains in a collection. The existing intraspecies heterogeneity in microbial taxa cannot be resolved in phylogenetic inner branches, which require the introduction of additional fingerprinting tools for selection. Other methods currently used can include the application of high-throughput chemotaxonomic profiling methods such as those based on the whole-cell fatty acid composition [33] and the use of MALDI-TOF MS protein profiles, a promising alternative to conventional identification techniques [34], and molecular fingerprinting techniques based on the random amplification of genome-conserved repetitive regions (AFLPs, RAPDS, and REP fingerprinting) [35–37]. The generation of rapid fingerprints based on the restriction pattern of amplified conserved sequences in polyketide

synthase or nonribosomal peptide synthetase biosynthetic systems provides additional information about the diversity and the biosynthetic potential of the new isolates [38].

3.3. Cultivation and Extraction. Traditionally the generation of microbial product libraries was based on the empiric cultivation of microbial strains in several nutritional conditions using different liquid and solid formats in varying volumes and by extracting the fermentation broths to generate crude extracts or semipurified fractions containing mixtures of specialized metabolites. The use of a limited number of three to four conditions at once, employing different media compositions, cultivation formats, or incubation periods or temperatures, was generally accepted as being sufficient to produce new, specialized metabolites, without real knowledge of the nutritional requirements and physiology of most of the groups of strains being screened and the key elements involved in regulating their specialized metabolite production. Nowadays, the continuously increasing number of whole-genome sequences of known producers shows that a large fraction of the genome remains silent and that switching on cryptic pathways might trigger the production of novel molecules [39–42]. The OSMAC (one strain, many compounds) approach has been proposed as an alternative way of exploring each strain in multiple conditions to better exploit their specialized metabolism and to trigger part of this microbial biosynthetic potential [43]. The use of multiple nutritional conditions has recently been explored by many groups to generate large screening extracts libraries in different formats (tubes, flasks), but miniaturized, parallel fermentation in deep-well plates represented a major breakthrough in the scale and numbers of conditions that can be tested [44]. All major taxonomic groups of actinomycetes and filamentous fungi can be cultivated in a large variety of complex and synthetic liquid media of diverse composition in carbon sources, inorganic or complex nitrogen sources, trace elements, and phosphate-controlled levels [20]. By testing in parallel a high number of nutritional conditions, minor groups of isolates can be explored and screened for the production of antibiotic activities. Identifying production media that can further promote their microbial biosynthetic potential increases the chances of producing novel molecules and identifying active extracts that can be then pursued on a larger scale in chemical isolation projects [45, 46].

The production of specialized bacterial metabolites can be stimulated by using known chemical inducers (e.g., siderophores, rare earths, or metabolism intermediates) [47–50], small, diffusible, bacterial, hormone-like molecules such as the γ-butyrolactones, and other butenolides [51]. Other elicitors of specialized metabolism include N-acetyl-glucosamine that when added to production media modulates the N-acetyl-glucosamine-responsive protein DasR [52] or generating ribosomal mutations that result in altered ppGpp biosynthesis and catabolite repression that favor biosynthesis [53]. Epigenetic modulation of fungal expression by histone acetylation and methylation has a strong influence on antibiotic production [54], and small-molecule epigenetic inhibitors of histone deacetylase (HDAC) or DNA methyltransferase (DMAT) are used to activate silent, natural product pathways in different fungal species [55, 56]. Similarly to fungi, HDAC inhibitors such as sodium butyrate or splitomicin have been reported to activate cryptic pathways in *Streptomyces coelicolor*, and HDAC orthologues have been identified to be broadly distributed in actinomycetes [57], offering new avenues to induce cryptic or poorly expressed specialized metabolites in these taxa and expand the chemical diversity of microbial product libraries.

Whereas the production conditions are key to promoting the biosynthetic potential, microbial product libraries comprise a collection of extracts and are also defined by the type of extraction used in their preparation. Extraction procedures should be designed to ensure the widest diversity of compound polarities in the extracts. These can range from simple whole-broth extraction with solvents of different polarity (from aqueous methanol or acetone miscible with the broth to more nonpolar solvents such as ethyl acetate or methyl-ethyl-ketone, providing cleaner extracts of mid-polarity compounds) to solid-phase extraction with ion exchange resins that directly enrich metabolites from the broth (cross-linked polystyrene Diaion HP20 or XAD resins) [58, 59]. Orthogonal fractionations that are used to generate prefractionated libraries reduce the complexity of the extracts, enabling screening at higher concentrations and simplifying the following dereplication phase [60].

3.4. Antibacterial Screening Assays. Antibiotic screening strategies of natural products have seen an important evolution in the past few decades, from the low-throughput, early phenotypic assays—used to identify compounds only targeting pathogens without any previous potential mode of action hypothesis—to high-throughput, whole-cell, target-based assays and structured-based design derived from *in silico* screening [61, 62]. High-throughput screening of microbial product libraries continues to be commonly based on phenotypic assays that have the advantage of utilizing intact bacteria and ensure that the active compound can penetrate the bacterial membranes and reach their target. Nowadays, these assays offer the possibility of integrating reporter genes to run whole-cell, target-based screens, in liquid- or agar-based format, including single- or two-plate assays, which aim to identify differential activity. The different types of assays targeting classical bacterial functions and essential pathways, including DNA replication, cell wall biosynthesis, and protein biosynthesis, have been extensively described in recent papers [63, 64]. Among these approaches, one of the breakthroughs is the use of *Staphylococcus aureus* genes essential for growth to develop a series of screens based on reducing the expression of targets to identify bacterial inhibitors. The induction of antisense RNAs to selectively decrease the production of intracellular gene products has been developed as a primary screening procedure for discovering new antibiotics [65] and was effectively employed to find novel classes of inhibitors with novel modes of action, such as the fatty acid synthesis inhibitors platensimycin and platencin, and a long list of new protein synthesis and protein secretion inhibitors [64]. An effective screening approach has consisted in the use of mechanism-based profiling using the *S. aureus* fitness test-based genome-wide screening for

upfront empiric evaluation of the antimicrobial activities derived from the screening of microbial product libraries on a wide panel of bacterial pathogens [66]. The *S. aureus* fitness test consists of a collection of inducible *S. aureus* antisense RNA strains engineered for reduced expression of a single target that corresponds to essential genes for which inducible antisense RNA expression determines a growth phenotype. This assay generates a profile of strain sensitivities specific for the mechanism of action (MOA) of the compound being tested and it has been used to profile and reveal novel activities in crude microbial product extracts [63].

3.5. Chemical Dereplication Process. Given that known molecules continue to be rediscovered in microbial product extracts, all the HTS screening strategies have been accompanied by the implementation of efficient, early LC-MS dereplication platforms to identify known compounds in natural products databases containing known antibiotic compound spectra [67]. For identification of the bioactive compounds in microbial products extracts, bioassay-guided fractionation and further purification of the active molecule from new, large-scale refermentation of the original microbial producer are required. To miniaturize the production conditions in HTS, the desired metabolites need to be reproduced in larger fermentation formats (tubes, flasks, and bioreactors; see the following section on fermentation) later on. After confirming the original hit activity in the new extract, several rounds of chromatographic separations following the biological activity in the enriched fractions ensure that the active component has been enriched. Analysis of the active fractions by LC-MS in each round of fractionation permits dereplication of any known components that can be recognized in reference natural product databases and explains the observed activity. Normally, three to four rounds of fractionation are needed to obtain the desired molecule as a pure compound with >95% purity [68, 69]. NMR and LC-MS analytical methods are then applied not only to assess the purity of the compounds but also to generate the dossier of spectra needed to elucidate the structure of the novel compounds [60].

4. Fermentation Is Often the Only Way to Produce Novel Natural Microbial Products

Antibiotics are the most important category of bioactive compounds extracted from microorganisms using fermentation. During the discovery process, which is based on biologically guided screening (see the section above), sufficient amounts of active fractions need to be produced by selected microbial strains for the initial biological profiling and to elucidate the chemical structure. During the development and clinical phases, the large-scale production of antibiotics from microbial fermentations is coupled with an intensive effort to improve the strain (see the section below) in order to reduce production volumes and costs and guarantee quality and reproducibility of the drug bulks. Later, when marketing the antibiotic, which is driven by profitability and competitiveness, lower operational costs with concurrently higher yields are required for microbial production [21]. To achieve that goal, manipulating and improving microbial strains and their growing conditions (upstream process) remain the main tools since any purification scheme (downstream) at this stage is hard to improve and change due to the rigorous manufacturing regulations.

For the majority of antibiotics, the only feasible supply process continues to be fermentation, total synthesis being too complicated or too expensive. Table 1 shows that the vast majority of the antibiotic drugs introduced into the market since 2000 are microbial products and are still produced by fermentation. Most natural products are so complex and contain so many centers of asymmetry that they probably will never be produced commercially by chemical synthesis. As an example, total chemical synthesis of the glycopeptide teicoplanin was performed by substantially inventing a new chemistry [70], but it is too expensive and microbial fermentation remains the only way to produce this valuable drug [71, 72]. However, compared to synthetic processes, manufacture by fermentation is more difficult to control; thus, it can lead to the formation of more variable antibiotic products with more complicated and less predictable composition and impurity profiles. This is due to the fact that (a) the purity of the active substances is dependent on the fungal or bacterial strains that produce the antibiotic; (b) the conditions under which strains are processed may vary; (c) the raw materials that are utilized, including the quality of water in which the strains grow, may also vary; and (d) the extraction and purification processes may have limited selectivity [73].

Hence, the crude product obtained by fermentation might not be a single antibiotic substance or entity, but rather a complex mixture of analogues, as is the case with teicoplanin (a complex of five related compounds designated teicoplanins $A_{2\text{-}1}$–$A_{2\text{-}5}$ characterized by five different linear or branched ten- or eleven-carbon fatty acids) [72], colistin (a multicomponent polypeptide antibiotic, comprised mainly of colistins A and B) [74], and gentamicin (oligosaccharide antibiotic composed of a mixture of three components designated as C′, C′a, and C2) [75]. Therefore, it might be difficult to compare apparently identical active ingredients unless they originate from the same manufacturer.

The need to improve the fermentation process (and reduce the cost of a multistep process) is particularly demanding for producing those natural scaffolds that undergo semisynthetic modification, as in the case of the second-generation glycopeptides (dalbavancin: trade name Dalvance, Durata Therapeutics; oritavancin: trade name Orbactiv, The Medicins Company; telavancin: trade name Vibativ, Theravance) recently approved by the Food and Drug Administration (FDA) [76].

4.1. Antibiotic Fermentation Process. Notwithstanding the key role of the fermentation process, not very much has changed since the first submerged fermentation process was developed to meet the demand for penicillins after the Second World War and the processes for producing antibiotics today are very similar to those employed 60 years ago. The fermentation process usually starts with a working cell bank (WCB) inoculated in a flask containing a vegetative medium (in which production does not occur) to allow the strain to grow. After a period that can vary depending on the strain, one or

Table 1: Examples of natural products (NP), semisynthetic modified natural products (SNP), natural product-derived but produced by chemical synthesis (NP-derived), or totally synthetic antibiotics (S) launched since 2000: production method, chemical class, activity against Gram-positive and/or Gram-negative bacteria, lead source, and producing organism.

Production	Class	NP-lead source	Lead source	Antibacterial spectrum	Drug name	Year approved
Chemical synthesis	Oxazolidinone		S	G+	Linezolid	2000
Fed-batch fermentation	Lipopeptide	Actinomycete (*Streptomyces roseosporus*)	SNP (A21978C)	G+	Daptomycin	2003
Chemical synthesis	Carbapenem		NP-derived	G+/G−	Doripenem	2005
Fed-batch fermentation	Pleuromutilin	Fungus (*Pleurotus* spp.)	SNP (pleuromutilin)	G+	Retapamulin	2007
Fed-batch fermentation	Glycopeptide	Actinomycete (*Amycolatopsis* spp.)	SNP (vancomycin)	G+	Telavancin	2009
Fed-batch fermentation	β-lactam	Fungus (*Cephalosporium acremonium*)	SNP (cephalosporin)	G+/G−	Ceftaroline fosamil	2010
Fed-batch fermentation	Tiacumicin	Actinomycete (*Dactylosporangium aurantiacum*)	NP	G+	Fixadomicin	2011
Fed-batch fermentation	Glycopeptide	Actinomycete (*Nonomuraea* sp.)	SNP (A40926)	G+	Dalbavancin	2014

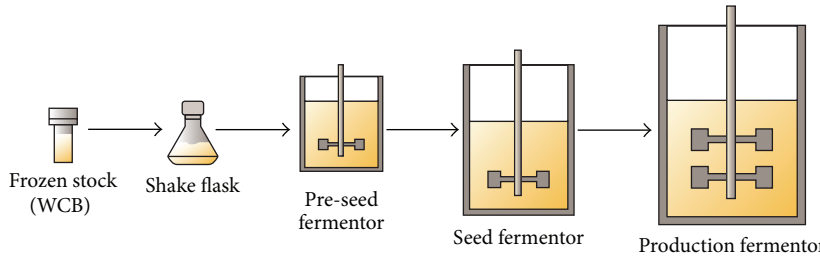

Figure 2: Flow diagram for the classical fermentation process: the number of seed steps may vary according to the final scale of the production fermentor.

a series of increasing volume reactors containing vegetative medium are serially inoculated to obtain enough material to start the last-vessel fermentation within the production medium (Figure 2). Submerged fermentations for producing antibacterials are usually performed in stirred tank reactors and are operated in batch or fed-batch mode. In batch reactors all components, except gaseous substrates such as oxygen, pH-controlling substances, and antifoaming agents, are placed in the reactor at the beginning of the fermentation.

Batch processes are simple and robust, but the only way to reach a high cell density is the fed-batch mode, which is more complex but allows the metabolism of the strain to be controlled [77]. In a fed-batch process, one or more nutrients are added in order to control the reaction rate according to its concentration, avoiding catabolite repression (see below) [77]. Most antibiotics are produced with the fed-batch system (e.g., teicoplanin [72], daptomycin [78], tylosin [79], and β-lactams [80]) (see Table 1). Continuous culture is not common in the pharmaceutical industry because the probability of mutation and contamination is higher. Scaling up the fermentation process usually constitutes the final step in any research and development program for large-scale industrial manufacture of fermentation products [81]. Production reactor sizes range from 40 to 100 cubic meters. It is important to understand that the process of scaling up a fermentation system is frequently governed by a number of important engineering considerations and is not simply a matter of increasing culture and vessel volume.

4.2. Regulation of Antibiotic Synthesis and Medium Composition. Antibiotics are usually not produced during the phase of rapid growth but rather are synthesized during a subsequent stationary phase. Antibiotic production starts when growth is limited after one key nutrient source is exhausted: carbon, nitrogen, or phosphate. For example, penicillin biosynthesis by *Penicillium chrysogenum* starts when there is no longer any glucose in the culture medium and the fungus starts consuming lactose, a less readily utilized sugar [82].

The main regulation effect in specialized metabolism is, in fact, carbon catabolite repression, defined as the control

(inactivation) of specific operons in favor of a primary and efficient utilization of a simple carbon source (commonly, but not always, glucose). The operons/genes/enzymes involved in crucial steps of biosynthesizing specialized metabolites are under catabolite repression. Catabolite repression is strictly linked to growth rate and growth phases since only after easily utilizable substrates have been consumed can the efficient production of specialized metabolites begin. Therefore, regulating metabolite biosynthesis ensures that precursors and metabolic energy are invested in the manufacture of specialized metabolites only under environmental circumstances and at developmental stages where those molecules contribute to the fitness of the organism [83, 84].

Glucose represses the production of many antibiotics (e.g., daptomycin [78], clavulanic acid [85], and aminoglycoside antibiotics such as streptomycin, kanamycin, neomycin, and gentamicin), but the molecular mechanism underlying glucose repression has resisted molecular analysis for a long time, although more recently this topic was thoroughly elucidated and widely covered in the literature [49, 84–87]. Readily utilizable nitrogen sources repress enzymes of specialized metabolism during the biosynthesis of cephalosporin [54, 88], cephamycin [89], tylosin [90], and erythromycin [91]. Similarly, free inorganic phosphate depletion from the growth medium is required to trigger production of tetracyclines [92, 93], β-lactams, and glycopeptides [93, 94]. Whereas the molecular mechanism for PhoP-mediated phosphate control is partially understood at the molecular level [93, 94], the signal sensors and signal transduction cascades involved in regulating metabolism by other stress factors need to be further elucidated [49].

To improve the production of antibiotics, slow-metabolizing carbon, nitrogen, and phosphorous sources are used: complex substrates such as polysaccharides (e.g., starch), oligosaccharides (e.g., lactose), and oils (e.g., soybean oil) are often preferred to glucose, and yeast extract, corn steep liquor, and soybean flour are commonly essential components for supplying nitrogen, phosphorous, vitamins, and trace elements to antibiotic-producing strains. In media containing a mixture of rapidly used carbon, nitrogen, and phosphorous sources and slowly used sources, the former are used first to produce cells and the latter employed once the rapidly assimilated compounds are depleted to sustain the production of specialized metabolites during the stationary phase of growth. Recent examples of how optimization of medium composition contributes to improving the final product concentration, yield, and volumetric productivity have been reported on daptomycin, nisin, cephalosporin C, clavulanic acid, and A40926, the precursor of semisynthetic dalbavancin [72, 78, 95–100]. In the case of daptomycin produced by *Streptomyces roseosporus* NRRL11379, Ng and coworkers have successfully established a cost-effective medium and feedback-controlling approach by utilizing dextrin as the major carbon source in fed-batch fermentation [78]. For glycopeptide antibiotics such as A40926 and teicoplanin, optimized media and processes have recently been proposed [65, 98–100]. The increasing list of specialized metabolism elicitors and chemical inducers, such as siderophores, rare earths, metabolism intermediates, diffusible bacterial hormone-like molecules, and N-acetyl-glucosamine, epigenetic modulators that are being used to activate cryptic or silent gene clusters during the screening processes (see previous paragraph on cultivation and extraction), can be also added to the production media to improve antibiotic production [47–57]. Limits in their use during scaling up of the fermentation process and product development consist in their cost and in the risk of chemical cross-contamination during the purification phase (downstream). Recent molecular studies have provided new insight into the role of catabolite carbon control. They demonstrated a relationship between antibiotic production and morphological development involving N-acetyl-glucosamine, which, when added to production media, modulates the N-acetyl-glucosamine-responsive protein DasR and pleiotropic regulation of both antibiotic synthesis and spore formation [52]. Molecular investigations also elucidated the role of ribosomal/RNA polymerase mutations resulting in altered ppGpp biosynthesis and in stringent response interplaying with catabolite repression [49, 101]. A thorough understanding of how global regulators (see section below on strain improvement) respond to a variety of nutritional or environmental stress signals, for example, phosphate, carbon, nitrogen starvation, heat shock, pH stress, and cell wall damage, is currently providing a more rational approach for defining medium and process conditions for antibiotic production [49, 91, 93].

5. Strain Improvement in the Postgenomic Era

With the development and advent of genome sequencing technologies [102, 103], it became obvious that most bacterial genomes contain a hidden wealth of clusters responsible for the biosynthesis of potential bioactive compounds [39–41] that await discovery. The main reason for the existence of such a plethora of undiscovered biosynthetic pathways is that many gene clusters are dormant or not expressed in sufficient quantities to be detected under typical fermentation conditions [104–106]. As discussed above, this is related to the existence of tight regulatory networks that precisely orchestrate specialized metabolite production in bacteria and respond to different environmental and intracellular signals [49, 86, 107]. Undoubtedly, a low yield of natural products represents a serious hurdle on the way to commercial production. Therefore, exploring and understanding the interplay between antibiotic production, regulatory networks, environmental and intracellular signals will provide us with keys to understanding specialized metabolite overproduction.

Nowadays, numerous strategies for improving strains have been and continue to be developed. Classical approaches for strain improvement were based on recursive rounds of mutagenesis and further selection [108, 109]. Despite the drawbacks (unwanted mutations and being time consuming and laborious), this strategy was successful and widely used for rapidly increasing the production yield of antibiotic-producing microbes. Most of the industrial overproducers currently in use were developed in this way [110, 111]. However, with the development of molecular biology, biotechnology, bioinformatics, sequencing technologies, and synthetic biology, new strategies have come to the scene and provide the opportunity for rational strain improvement (Figure 3).

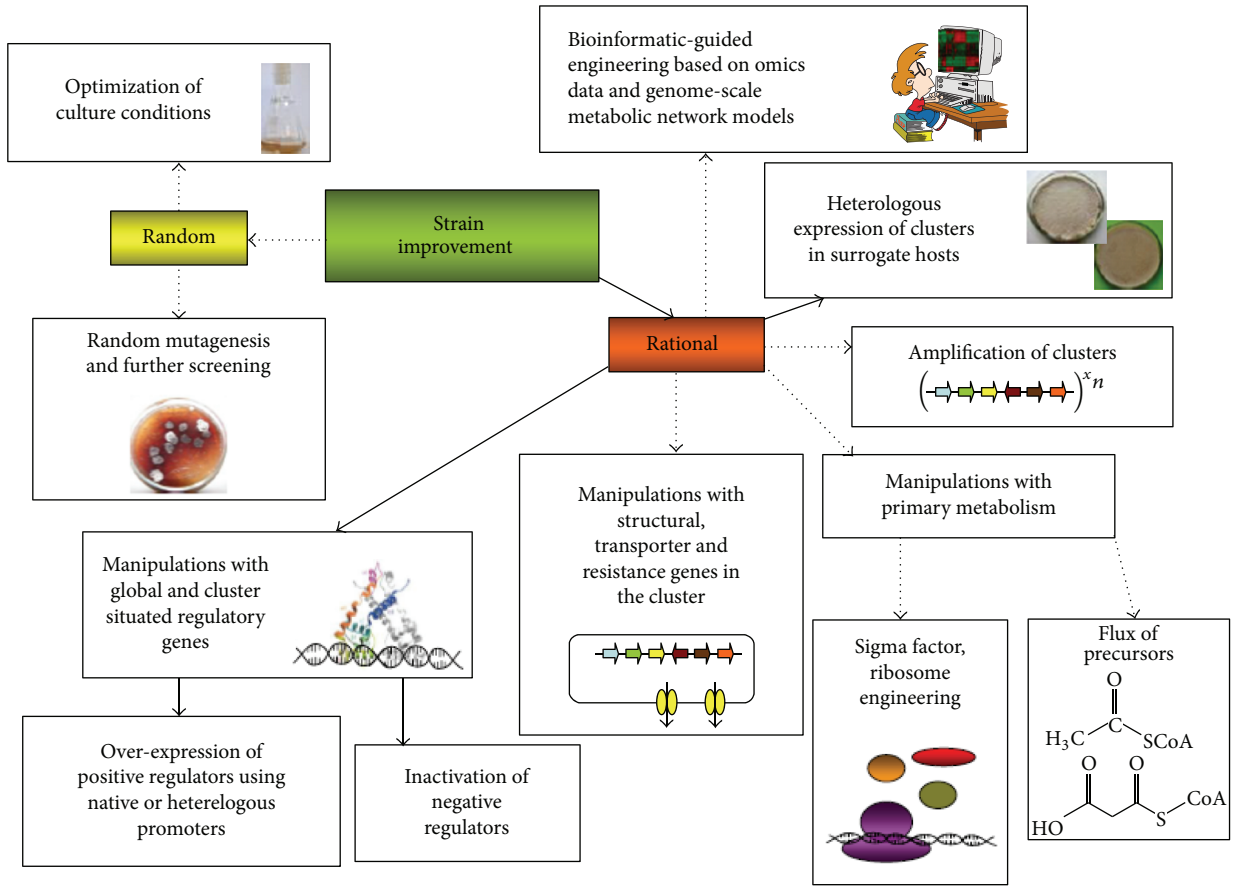

FIGURE 3: Approaches used for improving secondary metabolite production in actinobacteria. Solid arrows indicate strategies described in this review; dash-dotted arrows denote other strategies that are used.

Overall, all of these relatively new approaches are based on spatial, temporal, and quantitative regulation of gene expression at the transcriptional or translational level, or both, thereby enabling production of higher amounts of specialized metabolites by overcoming bottlenecks, optimizing expression of genes, and redirecting the flux of precursors. Therefore, titer can be elevated by overexpressing positive regulators or deleting repressors [94, 104, 112, 113]; amplifying gene clusters [114]; redirecting the flux of primary metabolites and precursors [104–115]; overexpressing structural genes that constitute bottlenecks on the way to metabolite production [116, 117]; manipulating resistance genes and transporters responsible for the flux of antibiotic [118–120]; ribosomal engineering [101, 105]; and so forth. Substituting native promoters in a cluster with well-defined, strong promoters, either constitutive or inducible, gives an opportunity to bypass existing regulatory machinery of the host strain and improve production [121, 122]. In some cases appreciable yields of metabolites can be obtained by expressing gene clusters in surrogate hosts which are easy to manipulate (*Streptomyces lividans, Streptomyces albus*) or which are industrial strains (*Streptomyces avermitilis*) or which are genetically engineered, versatile hosts with reduced genomes (*S. avermitilis* SUKA, *Streptomyces coelicolor* M1154) [123–125]. In the following section of the review, only some examples of using regulatory genes, promoters, and heterologous hosts for rational strain improvement will be reported. Many superb and in-depth reviews have been published recently that describe different approaches for metabolic engineering of actinobacteria [104, 112, 113, 126, 127]. We refer the readers to them for a further comprehensive introduction to these topics.

5.1. Regulatory Genes as Basic Keys to Metabolite Overproduction. Genes involved in the production of antibiotics are located together on a chromosome or plasmid and form biosynthetic clusters. Such clusters usually contain structural, resistance, transporter, and regulatory genes. Therefore, regulatory genes that are associated with cluster and control biosynthesis of certain compound are named pathway-specific or cluster-situated regulators (CSR). They form the lowest level in the hierarchically organized regulatory network of antibiotic production in bacteria [49]. Since production of specialized metabolites is tightly connected to morphological differentiation and depends on a plethora of environmental conditions, expression of CSRs hinges on a variety of other pleiotropic, higher-level regulators that sense and transmit signals to them. In turn, CSRs, which are usually final checkpoints, transfer these signals to structural genes and switch biosynthesis of natural products on and off [49, 86]. However, like for every rule, there are exceptions

in the structure of biosynthetic gene clusters. Elucidation of the genetic organization of numerous biosynthetic pathways revealed that there are some which lack CSRs [128, 129]. These findings indicate that the cluster-situated layer of regulation is not mandatory and is absent in some clusters. In such clusters, the expression of structural genes is controlled by ubiquitous regulatory genes that occupy higher levels in the regulatory web [128, 129].

According to how specialized metabolite production is influenced, all regulators can be conventionally classified into two groups: positive regulators, which activate, and negative regulators, which repress the biosynthesis of natural products. With the aim of enhancing the titer, both pleiotropic and CSRs, native and heterologous ones, are used. CSRs usually give an opportunity to manipulate one biosynthetic pathway, whereas global regulators might affect production of several specialized metabolites and/or morphological differentiation. Therefore, the effect of a pleiotropic regulatory gene very often depends on its position in the hierarchically organized regulatory network and in some cases might be unpredictable.

5.2. Manipulations with Positive Cluster-Situated Regulatory Genes. Overexpression of positive, pathway-specific regulators mainly enhances the transcription of structural genes responsible for the production of certain metabolites and therefore is a commonly used, single-step strategy for improving antibiotic yield. Herein, we will describe examples demonstrating the effectiveness of this approach for rational strain improvement.

Streptomyces globisporus 1912 is used to produce the angucycline antibiotic landomycin E (LaE). The landomycin biosynthetic gene cluster contains only one regulator gene, *lndI*, whose product is highly similar to the OmpR-PhoB subfamily of proteins. By inactivating it, antibiotic production was prevented in the I2-1 mutant, which confirms the role of LndI as an activator of LaE biosynthesis. Complementation of the I2-1 mutant with three additional copies of *lndI* gene resulted in 15-fold increase in LaE production in comparison to the wild-type strain 1912 [130], demonstrating the effectiveness of such an approach for improving the strain.

Simocyclinone D8 is an aminocoumarin compound that is produced by *Streptomyces antibioticus* Tü6040. *simReg1*, which belongs to the OmpR-PhoB subfamily of regulators, is one of three regulatory genes in the simocyclinone biosynthetic gene cluster. Its inactivation abolished antibiotic production, while overexpression of *simReg1* in an integrative pSET152-derived plasmid increased the simocyclinone titer 2.5-fold [131].

Other examples are as follows: (a) the C-1027 titer in *S. globisporus* was improved 5-fold after overexpressing the *sgcR1* gene, coding for a StrR-like protein [132]; (b) amplifying the *claR* gene encoding the LysR family protein in multicopy plasmids resulted in a threefold increase in clavulanic acid biosynthesis and in a sixfold increase in alanylclavam production [133]; (c) inserting a single copy of *pimM*, a LuxR type regulator, into the *S. natalensis* wild-type strain elevated pimaricin production 2.4-fold [134]; (d) overexpressing *fdmR1*, the encoding pathway-specific activator of the SARP family, led to a 5.6-fold increased production of fredericamycin A in *S. griseus* [135]; (e) amplifying the *tcp28* or *tcp29* genes, which encode StrR and LuxR family regulators, respectively, in the *Actinoplanes teichomyceticus* wild-type strain boosted teicoplanin production 1.5-3-fold [136, 137].

5.3. Manipulations with Negative Cluster-Situated Regulatory Genes. An effective and promising alternative method to overexpressing cluster-situated activators to boost antibiotic production is to inactivate pathway-specific repressors. This is exemplified by the disruption of the *lipReg3* gene coding for the MarR-type regulator that controls lipomycin export in *S. aureofaciens* Tü117, which led to a 4-fold improvement in lipomycin production in comparison to the wild-type strain [138].

Other examples that have proven the effectiveness of this strategy are as follows: (a) inactivation of the *jadR2* gene, coding a "pseudo" γ-butyrolactones receptor, in *S. venezuelae* generated the mutant that produces jadomycin without stress treatments (toxic concentration of ethanol, etc.) [139, 140]; (b) inactivation of another deduced γ-butyrolactone receptor coding gene *tylP* led to a 1.5-fold improvement in tylosin production in *S. fradiae* [141]; (c) deletion of the *ptmR1*, encoding GntR type repressor, in *S. platensis* MA7327 resulted in, on average, 100-fold overproduction of platensimycin and platencin compared to the wild-type strain [142]; and (d) inactivation of the TetR type regulator *alpW* in *S. ambofaciens* triggered constitutive production of kinamycin, a compound with antibacterial activity [143]. Thus, inactivation of repressor coding genes is useful not only for elevating antibiotic production, but, in some cases, for wakening silent gene clusters.

5.4. Manipulations with Pleiotropic Regulatory Genes. Successful application of omnipresent positive pleiotropic regulators to improve the titer of compounds whose biosynthetic gene clusters contain CSRs, or which are free of them, has also been shown. In most cases, a positive effect of their overexpression is due to the activation of cluster-situated regulatory gene expression or direct activation of the expression of structural genes in the cluster. For instance, overexpression of the pleiotropic regulator *afsRsv* in *S. venezuelae, S. peucetius*, and *S. lividans* TK24 led to a 4.85-, 8-, and 1.5-fold increase in pikromycin, doxorubicin, and actinorhodin production, respectively, relative to the wild type [144]. In the case of *S. venezuelae*, the increase in pikromycin production was caused by enhanced expression of the pathway-specific regulator gene *pikD* and the ketosynthase gene [144]. By introducing additional copies of the *afsR* or *afsS* genes into *S. coelicolor*, actinorhodin production could also be increased [145].

Streptomyces ghanaensis is a producer of phosphoglycolipid antibiotic moenomycin A [146]. The moenomycin biosynthetic gene cluster does not contain CSRs; therefore, different pleiotropic regulators were used to improve the moenomycin titer. Overexpression of the *adpAgh* gene, a pleiotropic regulator of antibiotic production and morphological development, led to a 2.5-fold improvement in moenomycin production in *S. ghanaensis* compared to the wild-type strain [129]. Introduction of the second copy of *bldAgh*, a leucyl

tRNA coding gene, into the wild-type strain *S. ghanaensis* led to a 1.6-fold increase in moenomycin production [129]. Overexpression of the *relA*, a ppGpp synthetase gene from *S. coelicolor*, led to a 2-fold improvement in moenomycin production in *S. ghanaensis* relative to the wild type [147].

Similarly, inactivation of negative pleiotropic regulators in *S. ghanaensis* increased moenomycin production. The gene *absB* codes for the RNAseIII endoribonuclease involved in global regulation of morphological differentiation and antibiotic production in *S. coelicolor* [148]. By deleting it, moenomycin production was improved 2.7-fold compared with the parental strain [129]. Inactivation of another global regulator gene, *wblA(gh)*, encoding a homologue of the WhiB-family of proteins, produced a 2.3-fold increase in moenomycin biosynthesis in *S. ghanaensis* [149].

Disruption of the (p)ppGpp synthetase gene, *relA*, in *S. clavuligerus* boosted clavulanic acid production 3- to 4-fold and that of cephamycin C increased about 2.5-fold [150], confirming that there might be a pleiotropic effect of global regulator amplification or inactivation.

5.5. Promoters as Bio-Bricks for Titer Improvement. Another common metabolic engineering approach to induce or enhance the expression of silent or poorly expressed pathways is based on replacing native promoters in a cluster with well-defined, strong promoters, decoupling the metabolic pathway from the existing cellular regulatory networks. Examples described below clearly prove the effectiveness of the combination of two metabolic engineering strategies: amplification of positive regulators and their expression under the control of heterologous promoters of various strengths.

For this purpose different natural or synthetic constitutive or inducible promoters may be used [137, 151]. One of the most widely employed promoters in streptomycetes is the erythromycin resistance gene *ermEp* from *Saccharopolyspora erythraea* or its upregulated variant *ermEp** [152]. For example, simultaneous overexpression of the *dnrN*, *dnrI*, and *afsR* regulatory genes under the control of *ermEp** in *S. peucetius* led to a 4.3-fold increase in doxorubicin production [153]. Another prominent example of the use of this promoter is the improvement in tylosin production in *S. fradiae*. Biosynthesis of tylosin is orchestrated by the complicated interplay of five regulators [154]. To bypass existing regulatory network-positive regulators, *tylS* or *tylR* was placed under the control of the *ermEp** and overexpressed in the *S. fradiae* wild-type strain. This boosted tylosin production 3.8- and 5.0-fold, respectively [154]. Production of teicoplanin in the nonstreptomycetes actinomycete *A. teichomyceticus* was improved 2.8-fold and 10-fold by overexpressing the StrR-type regulator *tcp28* under the control of the promoter of the SARP regulator gene *actII-ORF4* and apramycin gene resistance promoter (*aac(3)IVp*), respectively, which appeared to be stronger in this strain than the widely used *ermEp* [136, 137]. This reflects the necessity to test the activity of heterologous promoters in a particular strain since their activity might differ in various species. Therefore, the repertoire of available promoters should be extended.

5.6. Heterologous Expression of Clusters as a Way to Overproduction. With the advent of genome sequencing and metagenomics, a plethora of clusters coding for putative biologically active compounds which previously eluded discovery because of silencing or low product yield have become and continue to become available. In addition, there are growing numbers of actinobacteria that are difficult to culture and to manipulate genetically but which produce or might produce interesting chemical compounds. The reasons outlined above drove the development of a new approach in metabolic engineering for developing surrogate high-producing hosts for the heterologous expression of gene clusters. There are a number of potential surrogate hosts. Some of them derive from well-studied *Streptomyces* strains such as *S. lividans, S. coelicolor*, or *S. albus*; others are obtained from industrial strains or are genetically engineered, versatile hosts with reduced genomes. However, the main aim of this approach is still relevant and aims to build an ideal and universal surrogate host that will be easy to genetically manipulate, is fast growing and devoid of competitive sinks of carbon and nitrogen and antibiotic activity, and will be suitable for overproduction of different specialized metabolites.

To improve moenomycin production, several *Streptomyces* strains were used as heterologous hosts, namely, *S. coelicolor* M145, M512 (Δ*actII-ORF4*, Δ*redD*), *S. lividans* TK24, 1326, *S. albus* J1074, *S. venezuelae* ATCC10712, and *S. thermospinosisporus* NRRL_B24318. The highest moenomycin titer was found in *S. albus* strains, the lowest in *S. coelicolor* [147]. These data show a high variation between different hosts. Worthy of note is that the yield of antibiotic in *S. albus* was on average 4 times higher than in the native producer *S. ghanaensis* [147].

There are several genetically engineered heterologous hosts that were obtained by controlled minimization of genomes. For example, *S. coelicolor* M1154 was constructed by deleting four gene clusters (actinorhodin, prodiginine, calcium-dependent antibiotic, and cryptic polyketide) and subsequently introducing point mutations in the *rpoB* and *rpsL* genes that enhance specialized metabolite production [125]. Expression of the gene clusters for chloramphenicol or congocidine in this strain led to a 40- and 30-fold increase in production, respectively, in comparison to the *S. coelicolor* M145 strain. Another surrogate host was developed on the basis of the industrial strain *S. avermitilis* [124]. A region of more than 1.4 Mb that contains nonessential genes and gene clusters was deleted stepwise from the chromosome of *S. avermitilis*. Expression of cephamycin C, streptomycin, and pladienolide biosynthetic gene clusters was tested in the obtained strains. Production level of streptomycin in SUKA5 strain was approximately 3 times higher than in the native producer. Biosynthesis of cephamycin C was also greatly improved. However, the biosynthesis was switched on only in the presence of the activator CcaR. Substitution of the native promoter of the *ccaR* gene with the alternative *rpsJ* promoter led to an additional increase in cephamycin C production [124], underscoring the urgency and need to use the approaches outlined above to further improve antibiotic

production in genetically engineered heterologous hosts. The production of pladienolide in *S. avermitilis* engineered strains was also higher than in the *S. avermitilis* wild type [124].

Attempts to use well-studied, fast-growing, easy-to-manipulate, versatile, and widely used heterologous host such as *Escherichia coli* for the expression of actinobacterial gene clusters have also been made. This is exemplified by the production of the important antibacterial drug rifamycin. The starter unit for the RifA megasynthases is 3-amino-5-hydroxybenzoic acid (AHBA). The latter requires seven genes for biosynthesis, which are present in the rifamycin gene cluster [155]. First of all, the ability to synthesize the AHBA intermediate was reconstituted in *E. coli* BAP1. Afterwards, RifA was expressed in the AHBA-producing strain in the form of two bimodular proteins. As a result, the rifamycin intermediate P8_1-OG was synthesized at a quantity of 2.5 mg/L [155]. Other attempts to express erythromycin and oxytetracycline gene clusters in *E. coli* have also been described [156, 157]. Despite several successful tries, numerous attempts to overexpress *Streptomyces* gene clusters in *E. coli* failed. Currently, the main obstacles on the way to the desired metabolites in *E. coli* are high GC content of genes, absence of starter and extender units necessary for production, and differences in regulatory networks that generate an inability to effectively transcribe heterologous pathways. However, despite these drawbacks and taking into account a number of advantages, *E. coli* continues to be an appealing host for heterologous expression of actinobacterial gene clusters.

6. Conclusions

Despite the diverse classes of antibacterials that have been discovered from microbial natural product screening, there is an urgent medical need for novel molecules endowed with novel mechanisms of action to counteract emerging and multiresistant Gram-positive and Gram-negative pathogens. The microbial diversity at the origin of these novel drugs will continue to guarantee those unprecedented chemical characteristics and biological properties that did not emerge from screening libraries of synthetic compounds. Classical biological activity-based screening for novel antibacterials also relies on previous knowledge of the ecology and genome information of microbial isolates to assess their potential to produce different compounds under different cultivation conditions. Fermentation media and other parameters are being changed, taking into consideration knowledge-based use of different elicitors and tailored carbon, nitrogen, and phosphorous sources. The dramatic advances made in exploring and understanding the interplay between antibiotic production, regulatory networks, and environmental and intracellular signals are now providing us with keys to discover and overproduce new antibiotics. Currently, a wide range of genetic engineering approaches offer a large choice of tools for rational strain and fermentation improvement that might speed up the discovery and development of new, effective drugs. Combination of a growing body of knowledge in modern technologies, such as whole-genome sequencing, transcription, and metabolite profiling, offers the opportunity to make bioinformatics-based predictions of possible ways for discovering and improving specialized metabolites. Undoubtedly, further developments in functional genomics and other analytic techniques that lead to the discovery of many new signal transduction pathways and new transcription factors will reveal new, attractive targets for strain improvement approaches in the near future. In addition, approaches used in metabolic engineering continue to provide an excellent basis not only for creating overproducers, but to ensure further exploration and exploitation of the hidden part of microbial wealth. The main goal today is to develop a suite of technologies that could be used to induce the production of cryptic metabolic genes and identify previously unreported molecules, with sufficient yields to overcome one of the major problems of this century: the lack of new antibiotics.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All the authors equally contributed to the review.

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Review Article

Biologically Active Metabolites Synthesized by Microalgae

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Microalgae are microorganisms that have different morphological, physiological, and genetic traits that confer the ability to produce different biologically active metabolites. Microalgal biotechnology has become a subject of study for various fields, due to the varied bioproducts that can be obtained from these microorganisms. When microalgal cultivation processes are better understood, microalgae can become an environmentally friendly and economically viable source of compounds of interest, because production can be optimized in a controlled culture. The bioactive compounds derived from microalgae have anti-inflammatory, antimicrobial, and antioxidant activities, among others. Furthermore, these microorganisms have the ability to promote health and reduce the risk of the development of degenerative diseases. In this context, the aim of this review is to discuss bioactive metabolites produced by microalgae for possible applications in the life sciences.

1. Introduction

Microalgae are unicellular microorganisms that grow in fresh or salt water and have varied shapes with a diameter or length of approximately 3–10 μm. The term microalgae includes prokaryotic and eukaryotic organisms [1]. Cyanobacteria and bacteria have very similar structural characteristics; however, they are classified as microalgae because they contain chlorophyll *a* and compounds related to photosynthesis. The so-called green algae are so named because of the presence of chlorophyll *a* and chlorophyll *b* in the same proportions as in higher plants [2].

Microalgae are photosynthetic organisms that play a key role in aquatic ecosystems. Approximately 40% of global photosynthesis is due to these microorganisms [3]. Microalgal metabolism reacts to changes in the external environment with changes in its intracellular environment. Thus, the manipulation of the culture conditions, or the presence or absence of certain nutrients, stimulates the biosynthesis of specific compounds.

Several studies have been conducted to investigate the products of microalgal metabolism not only to understand its nature but also to search for substances with possible applications to humans in different fields of interest. Screening of extracts or isolation of metabolites from different microalgae is a common method for determining the biological activity of these components. Microalgae have been described as rich sources of various biocompounds of commercial interest [4].

Bioactive compounds of microalgal origin can be sourced directly from primary metabolism, such as proteins, fatty acids, vitamins, and pigments, or can be synthesized from secondary metabolism. Such compounds can present antifungal, antiviral, antialgal, antienzymatic, or antibiotic actions [5]. Many of these compounds (cyanovirin, oleic acid, linolenic acid, palmitoleic acid, vitamin E, B12, β-carotene, phycocyanin, lutein, and zeaxanthin) have antimicrobial

Table 1: Principal bioactive compounds extracted from microalgae.

Microalgae	Bioactive compounds	Reference
Spirulina sp.	Polysaccharides	[133]
Spirulina platensis	Phycocyanin, C-phycocyanin, Phenolic acids, tocopherols (vitamin E), neophytadiene, phytol, PUFAs (*n*-3) fatty acids, oleic acid, linolenic acid, palmitoleic acid	[7, 39, 81]
Spirulina fusiformis	Diacylglycerols	[81]
Haematococcus pluvialis	Astaxanthin, lutein, zeaxanthin, canthaxanthin, lutein, β-carotene, oleic acid	[8, 39, 81]
Chlorella sp.	Carotenoids, sulfated polysaccharides, sterols, PUFAs (*n*-3) fatty acids	[7]
Chlorella vulgaris	Canthaxanthin, astaxanthin, peptide, oleic acid	[13, 39, 133]
Chlorella minutissima	Eicosapentaenoic acid (EPA)	[81]
Chlorella ellipsoidea	Zeaxanthin, violaxanthin	[133]
Dunaliella salina	*trans*-Betacarotene, *cis*-betacarotene, β-carotene, oleic acid, linolenic acid, palmitic acid	[12, 39, 81]
Dunaliella	Diacylglycerols	[81]
Botryococcus braunii	Linear alkadienes (C25, C27, C29, and C31), triene (C29)	[12]
Chlorella zofingiensis	Astaxanthin	[8]
Chlorella protothecoides	Lutein, zeaxanthin, canthaxanthin	[8, 102]
Chlorella pyrenoidosa	Lutein, sulfated polysaccharide	[39]
Nostoc linckia and *Nostoc spongiaeforme*	Borophycin	[81]
Nostoc sp.	Cryptophycin	[81]

antioxidant, and anti-inflammatory capacities, with the potential for the reduction and prevention of diseases [6–9]. In most microalgae, the bioactive compounds are accumulated in the biomass; however, in some cases, these metabolites are excreted into the medium; these are known as exometabolites.

Bioactive metabolites of microalgal origin are of special interest in the development of new products for medical, pharmaceutical, cosmetic, and food industries. Further research should be conducted with these bioactive compounds to verify their beneficial effects for humans, their degradability when released into the environment, and their effects when used in animals [4]. In this context, the aim of this review is to discuss bioactive metabolites produced by microalgae for possible applications in the life sciences.

2. Microalgae with Potential for Obtaining Bioactive Compounds

Microalgae are a group of heterogeneous microorganisms that have a great biodiversity of colors, shapes, and cell characteristics, and their manipulation is encompassed by the field of marine biotechnology. Among the thousands of species of microalgae believed to exist, only a small number of them are retained in collections around the world, and it is estimated that only a few hundreds are investigated for compounds present in their biomass. Of these, only and a few are industrially cultivated [9]. This untapped diversity results in potential applications for these microorganisms in several biotechnological fields, such as the production of biocompounds used in food, medicine, cosmetics, and pharmaceuticals and even in the energy industry [10].

Microalgae are a natural source of highly interesting biologically active compounds. These compounds have received much attention from researchers and companies in recent years due to their potential applications in different life science fields. The applications range from the production of biomass for food and feed to the production of bioactive compounds for the medical and pharmaceutical industries [9]. Considering the enormous biodiversity of microalgae and recent developments in genetic engineering, this group of microorganisms is one of the most promising sources for new products and applications [7].

Microalgae are autotrophic microorganisms that use light energy and inorganic nutrients (carbon dioxide, nitrogen, phosphorus, etc.) to develop and synthesize biocompounds that have high aggregated nutritional value and therapeutic functions, such as lipids, proteins, carbohydrates, pigments, and polymers. Recent studies have reported that microalgae can produce different chemical compounds with different biological activities, such as carotenoids, phycobilins, polyunsaturated fatty acids, proteins, polysaccharides, vitamins, and sterols among other chemicals [8, 11, 12].

Components of microalgal origin with antimicrobial, antiviral, anticoagulant antienzymatic, antioxidant, antifungal, anti-inflammatory, and anticancer activity, among others, were identified [13–18]. The study of the extraction of bioactive compounds from various microalgae, such as *Arthrospira (Spirulina), Botryococcus braunii, Chlorella vulgaris, Dunaliella salina, Haematococcus pluvialis, and Nostoc* (Table 1), has been investigated [12, 19, 20].

Figure 1: *Spirulina* sp. LEB 18 from LEB/FURG strains bank.

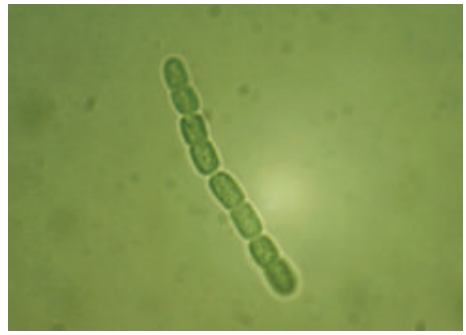

Figure 2: Microalga *Nostoc ellipsosporum* from LEB/FURG strains bank.

2.1. Spirulina. *Spirulina* (*Arthrospira*) is prokaryotic cyanobacteria (Figure 1) that belongs to Cyanophyta, which arose more than 3 million years ago, forming the current oxygen atmosphere, and has been important in the regulation of the planetary biosphere [21]. In 1981, *Spirulina* was approved by the FDA (Food Drug Administration) by the issuance of a GRAS (generally recognized as safe) certificate. The FDA has stated that *Spirulina* can be legally marketed as a food or food supplement without risk to human health [22].

Spirulina has a high protein value and high digestibility and contains significant amounts of essential polyunsaturated fatty acids and phenolic compounds [23]. Due to properties such as its high nutritional value and the presence of active biocompounds, this microorganism is one of the most studied microalgae worldwide [24]. The *Spirulina* protein content ranges from 50 to 70% (w/w) of its dry weight, the carbohydrate content from 10 to 20% (w/w), and the lipid content from 5 to 10% (w/w).

This microalga is rich in vitamins B1, B2, B12, and E (especially vitamin B12). Furthermore, *Spirulina* has a high content of pigments, minerals, and oligoelements (approximately 6 to 9% (w/w) biomass dry weight), of which the most important are iron, calcium, magnesium, phosphorus, and potassium [22]. Some studies have demonstrated the use of this microalga for the production of pigments due to its antioxidant properties [25–27]. β-Carotene represents approximately 80% of the carotenoids present in *Spirulina,* and other components, such as tocopherols, phycocyanin, and phycoerythrin, are also part of its composition [13]. Table 2 shows some of the bioactive compounds that have been extracted from *Spirulina*.

Cyanobacteria are known to produce intracellular and extracellular metabolites with potential biological activities, such as antibacterial, antifungal, antiviral, antitumor, anti-HIV, anti-inflammatory, antioxidant, antimalarial, herbicidal, and immunosuppressant effects [13, 28, 29]. The therapeutic importance of *Spirulina* has been reported in several studies. These include its use in the treatment of hyperlipidemia, cancer, HIV, diabetes, obesity, and hypertension, the improvement of immune response in renal protection against heavy metals and drugs, and the reduction in serum levels of glucose and lipids, among others [23, 27, 30, 31].

The world's largest producer, Hainan Simai Pharmacy Co. (China), annually produces 3000 tonnes of *Spirulina* biomass [13]. One of the largest industries in the world is Earthrise Farms (California, USA) (http://www.earthrise.com/). Many other companies market a wide variety of nutraceutical products produced from these microalgae. For example, the Myanmar *Spirulina* Factory (Yangon, Myanmar) produces pills, French fries, and pasta. Cyanotech (Hawaii, USA) produces and markets products under the name Spirulina Pacifica (http://www.cyanotech.com/). In Brazil, the Olson Microalgas Macronutrição company (Camaquã, Rio Grande do Sul) produces *Spirulina* sp. LEB 18 capsules for sale as a dietary supplement (http://www.olson.com.br/).

2.2. Nostoc. *Nostoc* is an edible microalga that belongs to the Nostocaceae group Cyanophyta that forms spherical colonies that link together as filaments. This microalga has heterocysts with a pattern of homogeneous cells and a regular distance between cells that compose the filament (Figure 2) [32]. The heterocysts fix atmospheric nitrogen for amino acid synthesis in the microalgal biomass. In the absence of a nitrogen source during microalgal cultivation, heterocysts form, avoiding the limitation of this nutrient for cell growth [33].

Nostoc microalgal biomass has been used in the medical field and as a dietary supplement because of its protein, vitamin, and fatty acid content. The medical value of this microalga was evidenced by its use in the treatment of fistula and for some forms of cancer [34]. Historically, the biomass of this microorganism is described as anti-inflammatory, and it also aids in digestion, blood pressure control, and immune boosting. Several studies suggest that *Nostoc* produces several compounds with antimicrobial, antiviral, and anticancer activity. These results have encouraged its cultivation on a large scale, and it has great economic potential due to its nutritional and pharmaceutical importance [35]. Table 3 presents some bioactive compounds that have been extracted from the microalga of the *Nostoc* genus.

Cyanovirin, a potential protein molecule produced by a *Nostoc* microalga, showed a positive effect in the treatment of HIV [36] and Influenza A (H1N1) [6]. *Nostoc* contains a spectrum of polyunsaturated fatty acids (PUFAs) that include essential fatty acids, such as linoleic, α-linolenic, γ-linolenic, octadecatetraenoic, and eicosapentaenoic acid [37]. Essential fatty acids are precursors of prostaglandins, engendering significant interest from the pharmaceutical industry.

Table 2: Bioactive compounds extracted from *Spirulina* genus.

Microalga	Bioactive compound	Concentration (%, w/w)	Reference
Spirulina fusiformis	C-phycocyanin	46.0	[8]
Spirulina platensis	C-phycocyanin	9.6	[8]
Spirulina platensis	Allophycocyanin	9.5	[8]
Spirulina sp.	C-phycocyanin	17.5	[8]
Spirulina sp.	Allophycocyanin	20.0	[8]
Spirulina platensis	Phenolic	0.71	[134]
Spirulina platensis	Terpenoids	0.14	[134]
Spirulina platensis	Alkaloids	3.02	[134]
Spirulina maxima	Phenolic	1.29	[121]
Spirulina maxima	Flavonoids	0.46	[121]

Table 3: Bioactive compounds extracted from the *Nostoc* genus.

Microalga	Bioactive compound	Concentration (%)	Reference
Nostoc sp.	Phycocyanin	20.0 (p/p)	[8]
Nostoc muscorum	Phenolic	0.61 (p/p)	[134]
Nostoc muscorum	Terpenoids	0.10 (p/p)	[134]
Nostoc muscorum	Alkaloids	2.30 (p/p)	[134]
Nostoc muscorum	Phycobilins	0.0229 (p/v)	[134]
Nostoc humifusum	Phenolic	0.34 (p/p)	[134]
Nostoc humifusum	Terpenoids	0.10 (p/p)	[134]
Nostoc humifusum	Alkaloids	1.65 (p/p)	[134]
Nostoc humifusum	Phycobilins	0.0031 (p/v)	[134]

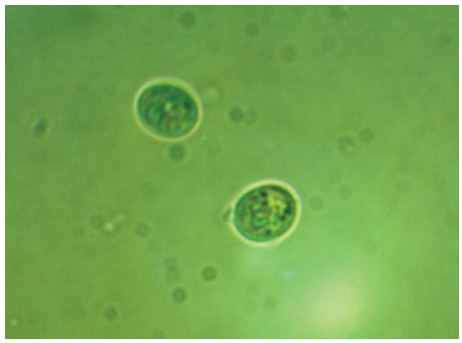

Figure 3: Microalga *Chlorella fusca* LEB 111 from LEB/FURG strains bank.

2.3. Chlorella. *Spirulina* and *Chlorella* represent the majority of the microalgal biomass market, with an annual production of 3,000 and 4,000 tons, respectively [38]. *Chlorella* sp. is a eukaryotic genus of green unicellular microalgae that belongs to the Chlorophyta group (Figure 3) [39].

This microalga was discovered by the Japanese, traditional consumers of algae, who usually enjoy it and use it as a food supplement. The microalga *Chlorella* is rich in chlorophyll, proteins, polysaccharides, vitamins, minerals, and essential amino acids. This microalga is 53% (w/w) protein, 23% (w/w) carbohydrate, 9% (w/w) lipids, and 5% (w/w) minerals and oligoelements [22].

These nutrient concentrations can be varied by manipulation of culture conditions. The biomass of this microalga is also rich in B complex vitamins, especially B12, which are vital in the formation and regeneration of blood cells. Like *Spirulina*, *Chlorella* has a GRAS certificate issued by the FDA and can thus be used as a food without risk to human health when grown in a suitable environment with proper hygiene and good manufacturing practices [22, 40].

Chlorella contains bioactive substances with medicinal properties. Experimental studies with *Chlorella* demonstrated their antitumor, anticoagulant, antibacterial, antioxidant, and antihiperlipidemia effects in addition to a hepatoprotective property and the immunostimulatory activity of enzymatic protein hydrolyzate [39, 41–44].

Many antioxidant compounds may be responsible for *Chlorella* functional activities. Antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid, and α-tocopherol, which are active against free radicals, were identified. Some of these compounds not only are important as natural colorants or additives but also may be useful in reducing the incidence of cancer and in the prevention of macular degeneration [39, 45] (Table 4).

The most important bioactive compound in *Chlorella* is β-1,3 glucan, an active immunostimulator that reduces free radicals and blood cholesterol. The efficacy of this compound against gastric ulcers, sores, and constipation has been reported. It also has been demonstrated to have preventive action against atherosclerosis and hypercholesterolemia,

Table 4: Bioactive compounds extracted from the microalgae of the *Chlorella* genus.

Microalga	Bioactive compound	Concentration (%, w/w)	Reference
Chlorella protothecoides	Lutein	4.60	[8]
Chlorella zofingiensis	Astaxanthin	1.50	[8]
Chlorella vulgaris	Phenolic	0.20	[134]
Chlorella vulgaris	Terpenoids	0.09	[134]
Chlorella vulgaris	Alkaloids	2.45	[134]
Chlorella minutissima	Phytol	2.70	[135]
Chlorella minutissima	Phenol	1.81	[135]

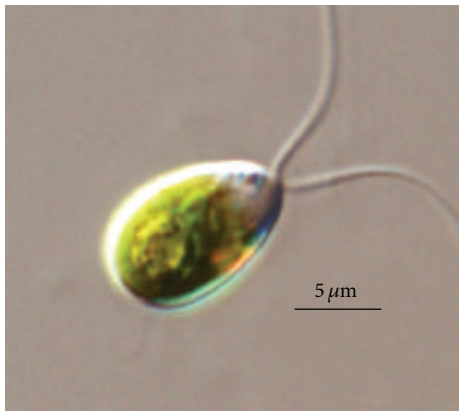

Figure 4: *Dunaliella* sp. microalga [48].

as well as antitumor activity [46]. *Chlorella* is produced by more than 70 companies. Taiwan Chlorella Manufacturing Co. (Taipei, Taiwan) is the world's largest producer of *Chlorella*, with over 400,000 tons of biomass produced per year (http://www.taiwanchlorella.com/index.php). Significant production also occurs in Klötze (Germany) (80–100 t yr^{-1} of dry biomass) [47].

2.4. Dunaliella. *Dunaliella* is a green unicellular halotolerant microalga that belongs to the Chlorophyceae group (Figure 4). This microalga is widely studied due to its tolerance of extreme habitat conditions, physiological aspects, and its many biotechnological applications. *Dunaliella* is a source of carotenoids, glycerol, lipids, and other bioactive compounds, such as enzymes and vitamins [48, 49].

This microalga is a major source of natural β-carotene, able to produce up to 14% of its dry weight under conditions of high salinity, light, and temperature as well as nutrient limitation [50]. In addition to β-carotene, this microalga is rich in protein and essential fatty acids, which can be consumed safely, as evidenced by GRAS recognition [22]. Table 5 presents some compounds that have been extracted from microalgae of the *Dunaliella* genus.

Compounds in the *Dunaliella* biomass have various biological activities, such as antioxidant, antihypertensive, bronchodilatory, analgesic, muscle relaxant, hepatoprotective, and antiedemal properties. The microalgal biomass can also be used directly in food and pharmaceutical formulations [22, 51].

Chang et al. [52] showed that *Dunaliella* cells contained antibiotic substances. According to these authors, the crude extract of this microalga strongly inhibited the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis,* and *Enterobacter aerogenes*. In another study, *Dunaliella* microalga also showed antibacterial activity against various microorganisms of importance to the food industry, including *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans,* and *Aspergillus niger* [49, 53].

Under ideal growing conditions, *Dunaliella* can be stimulated to produce approximately 400 mg of β-carotene per square meter of growing area. The cultivation of *Dunaliella* for the production of β-carotene has been conducted in several countries, including Australia, Israel, the USA, and China [54–56]. An ingredient of *Dunaliella* with a strong ability to stimulate cell proliferation and improve the energy metabolism of the skin was released by Pentapharm (Basel, Switzerland) [57]. New pilot plants are under development in India, Chile, Mexico, Cuba, Iran, Taiwan, Japan, Spain, and Kuwait [50].

3. Cultivation Conditions

The conditions for microalgal cultivation are important factors that influence the metabolism of these microorganisms, thus directing the synthesis of specific compounds of interest. Several researchers have noted the influence of incubation temperature, the pH of the medium, the period of cultivation, as well as salinity, light intensity, and medium constituents, on the synthesis of antimicrobial agents [58].

3.1. pH, Temperature, and Luminescence. pH adjustments are the primary measures used to prevent contamination by microorganisms, such as other microalgae species. pH control is also essential for effective absorption of the components of the culture medium because it directly affects the availability of various chemical elements [59]. The reduction of some nutrients in the culture medium can lead the producing of specific biocompounds. The difficulty of consuming a nitrogen source, for example, can lead microalgae to shift your metabolism for lipids or carbohydrates production [60].

Light is an indispensable factor for photosynthesis, causing the cells to reproduce and thereby increasing the cell

Table 5: Bioactive compounds extracted from the microalgae of the *Dunaliella* genus.

Microalga	Bioactive compound	Concentration (%, w/w)	Reference
Dunaliella salina	β-Carotene	12%	[8]
Dunaliella salina	All-*trans*-β-carotene	13.8%	[136]
Dunaliella salina	All-*trans*-zeaxanthin	1.1%	[136]
Dunaliella salina	All-*trans*-lutein	0.66%	[136]
Dunaliella tertiolecta	Sterols	1.3%	[50]
Dunaliella salina	Sterols	0.89%	[50]

concentration [61]. The illuminance also influences the biochemical composition of the biomass [62]. The fatty acid content can be reduced with increasing light incidence. This is because lipids are the major components of chloroplasts and the increased light energy demand greater activity of chloroplasts [63]. Studies also show the influence of illuminance on the microalgae antioxidants. According to Madhyastha [64], the application of blue light in the cultivation of the microalga *Spirulina fusiformis* through a phenomenon where the microalgae cells alter the sequence of amino acids with cysteine repeats enhanced the antioxidant capacity.

One of the most important factors for the growth of all living organisms is the temperature. The specific growth rate of the microalgae is directly correlated with the gross rate of CO_2 fixation/O_2 production (photosynthesis) and the respiration rate. Photosynthesis and respiration are temperature-dependent, with the respiration rate increasing exponentially with temperature [65]. Temperature has a great influence on the production of biomass, proteins, lipids, and phenolic compounds from microalgae. The optimum temperature for cultivation of microalgae is 35–37°C [66]. In studies conducted by Noaman [58] that were performed to verify which culture conditions stimulated the greatest production of antimicrobial agents by the microalga *Synechococcus leopoliensis,* it was observed that a temperature of 35°C and pH 8 produced a maximum concentration of this bioactive compound.

3.2. Bioreactors. Microalgae have attracted much interest for production of bioactive compounds, and in order to grow and tap the potentials of algae, efficient photobioreactors are required. A good number of photobioreactors can be used in production of various algal products [67]. Innovative cultivation systems and modification of biochemical composition of microalgae by simple changes in the growth media and cultivation conditions (nutrients, light intensity, temperature, pH, mixing, etc.) can lead to higher productivity of the targeted products [68].

Bioreactors can be classified as open or closed. Closed photobioreactors have attracted much interest because they allow a better control of the cultivation conditions than open systems. One of the major advantages of open ponds is that they are easier to construct and operate than most closed systems [67].

In open systems, temperature is a main limiting factor, as are variations in solar radiation that lead to low biomass concentrations. However, open systems are the most widely used due to their economic viability. Closed systems are generally used on a pilot scale for investigating problems related to economic viability. Furthermore, the use of closed systems is primarily used for microalgal species that do not grow in a highly selective medium, avoiding contamination of the cultures [69].

Closed bioreactors can provide high productivity, generating greater microalgal biomass per unit time. Other advantages of the use of closed bioreactors compared with open systems include the following: (i) virtually zero losses in connection with evaporation; (ii) a marked reduction of problems related to culture contamination by heterotrophic algae or other microorganisms; (iii) ease of biomass collection procedures due to smaller volumes of culture medium; (iv) greater control of gas exchange between the culture and the atmosphere; (v) a smaller occupied space; (vi) a high surface:volume ratio, which helps to increase the illumination of the system; and (vii) the possibility of obtaining high purity cultures [59].

3.3. Nutrients. The metabolism of microalgae can be autotrophic or heterotrophic. The former requires only inorganic compounds, such as CO_2, salts, and solar energy; the latter is not photosynthetic, requiring an external source of organic compounds for use as a nutrient and energy source. Some photosynthetic species are mixotrophic, having the ability to perform photosynthesis and use exogenous organic sources simultaneously [70].

Microalgae react to changes in their external environment with changes in their intracellular environment. Thus, the manipulation of the culture conditions or the presence or absence of nutrients stimulates the biosynthesis of specific compounds. This fact was first referenced by Richmond [71], who changed the composition of *Chlorella* biomass, particularly in their protein and lipid content, by varying cultivation conditions.

Noaman [58] found that leucine combined with citrate or acetate is the sources of nitrogen and carbon that produced higher concentrations of antimicrobial agents in the microalga *Synechococcus leopoliensis*. Coca et al. [72], studying the cultivation of *Spirulina platensis* in a medium supplemented with vinasse, obtained an increased protein yield compared to the unsupplemented culture medium. Ip and Chen [73], studying the cultivation of *Chlorella zofingiensis* under mixotrophic cultivation conditions, found that low concentrations of nitrate and a high glucose concentration favored the production of astaxanthin in this microalga.

Alonso et al. [74], studying the influence of nitrogen concentration in continuous cultivation on lipid concentration in *Phaeodactylum tricornutum*, noted that there was accumulation of saturated and unsaturated fatty acids when the nitrogen source was reduced.

Culture media are chemical preparations that are formulated to contain the nutrients necessary for the microorganisms to multiply and/or survive. The culture media should meet the nutritional needs of the microorganism, assist in process control, and have a reasonably fixed composition [75].

Among different microalgae, variations in the culture medium are mainly related to the amount of necessary nutrients. Even so, nutritional needs are dependent on environmental conditions [59]. Microalgae require macronutrients, such as C, N, O, H, P, Ca, Mg, S, and K, for their growth. The micronutrients that are generally required are Fe, Mn, Cu, Mo, and Co. Additionally, some species require lower concentrations of vitamins in the culture medium [76].

4. Advantages of Using Microalgae to Obtain Bioactive Compounds

Microalgae are important sources of bioactive natural substances. Many metabolites isolated from these microorganisms have shown biological activities and potential health benefits [77]. Microalgae accumulate specific secondary metabolites (such as pigments and vitamins) which are high value products that have applications in the cosmetic, food, or pharmaceutical industries [8, 78].

Microalgae live in complex habitats and are subjected to stress and/or extreme conditions, such as changes in salinity, temperature, and nutrients. Thus, these microorganisms must rapidly adapt to new environmental conditions to survive and thus produce a great variety of biologically active secondary metabolites that are not found in other organisms [79]. Some of the advantages of microalgal cultivation may be associated with taxonomic diversity, the diverse chemical composition, the potential for growth in a bioreactor under controlled conditions, and the ability to produce active secondary metabolites in response to the stress induced by extreme exposure conditions [39, 80].

In addition to their natural characteristics, other important aspects related to microalgae are the use of solar energy and carbon dioxide (CO_2) and a high growth rate which can produce higher yields compared to higher plants. In addition, microalgae can be grown in areas and climates that are unsuitable for agriculture; therefore, microalgae do not compete with arable food production land. The possibility of controlling the production of certain bioactive compounds by manipulation of culture conditions is another advantage of using microalgae [7, 81–83].

The cultivation of microalgae is a major mechanism for reducing excess carbon dioxide (CO_2) in the atmosphere by biofixation, in which an industrial process uses a CO_2-rich gas as a carbon source for microalgal growth. This mechanism contributes to a reduction of the greenhouse effect and global warming, further reducing the costs of the carbon source for growth, which is the greatest nutrient requirement for microalgae [13, 84].

The cultivation of microalgae is not seasonal; they are important for food in aquaculture systems and can effectively remove pollutants, such as nitrogen and phosphorus, from wastewater. Moreover, they are the most efficient solar energy biomass converters. Microalgae cultivation via sunlight-dependent systems contributes to sustainable development and natural resource management [13].

The integration of the production process of bioactive metabolites in a biorefinery is a sustainable means of energy production, food production, and the production of products with high added value [7]. The biorefinery concept based on microalgae depends on the efficient use of biomass through fractionation, resulting in several isolated products. This concept encompasses a biorefinery platform, which is capable of offering a wide variety of different products, such as products with applications in pharmaceuticals, medicine, food (protein, fiber), and biofuels [7, 85]. These benefits contribute to the economic viability of microalgal production [7, 8].

5. Bioactive Compounds

Bioactive compounds are physiologically active substances with functional properties in the human body. There is great enthusiasm for the development and manufacture of various biocompounds that can potentially be used as functional ingredients, such as carotenoids, phycocyanins, polyphenols, fatty acids, and polyunsaturated compounds [16].

An interest in the production of bioactive compounds from natural sources has recently emerged, driven by a growing number of scientific studies that demonstrate the beneficial effects of these compounds on health [80]. Natural products are important in the search for new pharmacologically active compounds. In general, they play a role in drug discovery for the treatment of human diseases [86]. Many clinically viable and commercially available drugs with antitumor and antiinfective activity originated as natural products.

Microalgae are a natural source of interesting biocompounds. Microalgae are known to produce various therapeutically effective biocompounds that can be obtained from the biomass or released extracellularly into the medium [11]. These microorganisms contain many bioactive compounds, such as proteins, polysaccharides, lipids, vitamins, enzymes, sterols, and other high-value compounds with pharmaceutical and nutritional importance that can be employed for commercial use [13].

5.1. Compounds with Antioxidant Function. Oxidative damage caused by reactive oxygen species to lipids, proteins, and nucleic acids can cause many chronic diseases such as heart disease, atherosclerosis, cancer, and aging. Epidemiological studies have demonstrated an inverse association between the intake of fruits and vegetables and mortality from diseases such as cancer. This phenomenon can be attributed to the antioxidant activity of these foods [87].

Microalgal biomass is considered a rich natural source of antioxidants, with potential applications in food, cosmetics, and medicine [87]. Antioxidant compounds, such as dimethylsulfoniopropionate and mycosporine amino acids, were isolated from microalgae and are potent chemical blockers of UV radiation [88]. In addition to these compounds, pigments, lipids, and polysaccharides with antioxidant activity can also be found in microalgal biomass.

Carotenoids and phycocyanins are the pigments most used in scientific research. C-phycocyanin (C-PC) is a blue photosynthetic pigment that belongs to the group of phycobiliproteins found in large quantities in the cyanobacteria, Rhodophyta, and Cryptophyte [89]. Phycocyanin has applications as a nutrient and natural food colorants and cosmetics. It is usually extracted from the biomass of *Spirulina* [90] and *Porphyridium cruentum* [91] and *Synechococcus* [89].

Among the carotenoid compounds, β-carotene and astaxanthin are prominent. These compounds have application in the food and pharmaceutical industries because of their antioxidant properties and pigmentation ability. In microalgal metabolism, they protect photosynthetic tissues against damage caused by light and oxygen [92]. *Dunaliella salina* is a microalga recognized as a major biological source of β-carotene pigment, producing more than 14% in dry biomass [46]. *H. pluvialis* is a source of the pigment astaxanthin, producing 1–8% of astaxanthin as dry biomass [93].

Polysaccharides represent a class of high value-added components with applications in food, cosmetics, fabrics, stabilizers, emulsifiers, and medicine [94]. Microalgal polysaccharides contain sulphate esters, are referred to as sulfated polysaccharides, and possess unique medical applications. The basic mechanism of therapeutic action is based on the stimulation of macrophages and modulation. The biological activity of sulfur polysaccharides is linked to their sugar composition, position, and degree of sulfation [95]. Among the microalgae capable of producing these compounds are *Chlorella vulgaris*, *Scenedesmus quadricauda* [96], and *Porphyridium* sp. [97].

5.2. Compounds with Antimicrobial Activity. The importance of discovering new compounds with antimicrobial activity is driven by the development of antibiotic resistance in humans due to constant clinical use of antibiotics. Microalgae are an important source of antibiotics with a broad and efficient antibacterial activity [11]. The antimicrobial activity of these microorganisms is due to the ability to synthesize compounds, such as fatty acids, acrylic acids, halogenated aliphatic compounds, terpenoids, sterols, sulfur-containing heterocyclic compounds, carbohydrates, acetogenins, and phenols [98].

The antimicrobial activity of extracts from microalgae is related to its lipid composition. The antimicrobial action of microalgae is also noteworthy because of the potential to produce compounds such as α- and β-ionone, β-cyclocitral, neophytadiene, and phytol [99]. Microalgae antimicrobial activity against human pathogens, such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis*, has been attributed to γ-linolenic acid, eicosapentaenoic acid, hexadecatrienoic acid, docosahexaenoic acid, palmitoleic acid, lauric acid, oleic acid, lactic acid, and arachidonic acid [99, 100].

The mechanism of action of fatty acids affects various structures in microorganisms; however cell membranes are the most impacted. Membrane damage most likely leads to a loss of internal substances from the cells, and the entry of harmful components reduces nutrient absorption, in addition to inhibiting cellular respiration. The ability of fatty acids to interfere with bacterial growth depends on both their chain length and the degree of unsaturation. Fatty acids with more than 10 carbon atoms apparently induce lysis of bacterial protoplasts [99].

Microbial polysaccharides and other biological compounds have antiviral and antimicrobial action. Microalgae produce extracellular sulfated polysaccharide (EPS) with acidic characteristics that has a potential as a therapeutic agent [101]. Highly sulfated antiviral polysaccharides from several species of microalgae consist mainly of xylose, glucose, and galactose. The EPS sulfate groups determine some characteristics of polysaccharides; it has been found that higher sulphate contents induced higher antiviral activities [94, 101]. The inhibitory effect of polysaccharides of microalgal origin is due to viral interactions or positive charges on the cell surface, thereby preventing penetration of the virus into host cells [99].

The cyanobacterium *Spirulina* (*Arthrospira*) can produce sulfated polysaccharides that have already found applications as antiviral agents, both *in vivo* and *in vitro* [102]. Eukaryotic microalgae, such as *Chlorella* sp. and *Dunaliella* sp., produce and secrete polysaccharides at relatively high levels [17]. The antibacterial ability of *Spirulina* has been correlated with their volatile composition, resulting in the identification of 15 elements, which constitutes 96% of total compounds. The major volatile components produced by *Spirulina* consist of heptadecane (40%) and tetradecane (35%) [39].

Some studies have reported that sulfated polysaccharides derived from microalgae inhibit viral infection, such as encephalomyocarditis virus, Herpes simplex virus types 1 and 2 (HSV1, HSV2), human immunodeficiency virus (HIV), hemorrhagic septicemia in salmonid virus, swine fever virus, and varicella virus [99, 103]. Carrageenan is a sulfated polysaccharide that can directly bind to human papillomavirus to inhibit not only the viral adsorption process but also the input and subsequent process of the uncoating of the virus [101].

5.3. Compounds with Anti-Inflammatory Action. Inflammation is an immediate reaction to a cell or tissue injury caused by noxious stimuli, such as toxins and pathogens. In this situation, the body recognizes the agents responsible for the attack and attempts to neutralize them as quickly as possible. Inflammation causes redness, swelling, heat, and pain, usually located at the site of infection [104]. Ingestion of anti-inflammatory compounds enhances the body's immune response and helps to prevent disease and aids the healing process. Microalgae produce several anti-inflammatory compounds in their

biomass that may exert a protective function in the body when consumed as food or used as pharmaceuticals and cosmetics.

Because of its anti-inflammatory capabilities, microalgal biomass is being considered for applications in tissue engineering for the development of scaffolds, for use in reconstitution of organs and tissues [105, 106]. This is an important application for humans, especially in patients with burns in which the skin was completely lost [107]. Among the most important microalgal compounds with such properties are long-chain polyunsaturated fatty acids (PUFAs) [108, 109], sulfurized polysaccharides [110], and pigments [111].

Many microalgal polysaccharides possess the ability to modulate the immune system through the activation of macrophage functions and the induction of reactive oxygen species (ROS), nitric oxide (NO), and various other types of cytokines/chemokines [112]. Macrophages are able to regulate several innate responses and secrete cytokines and chemocytokines that serve as signals for immune and inflammatory molecular reactions [113]. Sulfur polysaccharides with anti-inflammatory activity can be applied in skin treatments inhibiting the migration and adhesion of polymorphonuclear leukocytes [110]. Guzmán et al. [114] studied the anti-inflammatory capacity of the microalga *Chlorella stigmatophora* and *Phaeodactylum tricomutum* and concluded that both microalgae showed positive responses in the test of paw edema by carrageenan.

The PUFAs, especially $\omega 3$ and $\omega 6$ as eicosapentaenoic (EPA), docosahexaenoic (DHA), and arachidonic (AA) acids, have been applied in the treatment of chronic inflammation such as rheumatism and skin diseases [108]. Ryckebosch et al. [115] evaluated the nutritional value of the total lipids extracted from different PUFAs produced by microalgae. In this study, the microalgae *Isochrysis*, *Nannochloropsis, Phaeodactylum, Pavlova,* and *Thalassiosira* produced $\omega 3$ PUFA as an alternative to fish oil in food.

Among the pigments with anti-inflammatory activity, fucoxanthin carotenoid found in diatoms [116, 117] is capable of stimulating apoptosis in human cancer cells [118]. A phycocyanin, found in cyanobacteria, has an anti-inflammatory activity that occurs through the inhibition of histamine release [111, 119].

5.4. Compounds with Potentiality over Degenerative Diseases. In humans, the oxidation reactions driven by reactive oxygen species (ROS) can lead to irreversible damage to cellular components, including lipids, proteins, and DNA degradation and/or mutation. Consequently, this damage can lead to several syndromes, such as cardiovascular disease, some cancers, and the degenerative diseases of aging [120].

Chronic age-related diseases involve oxidative stress and inflammation and their consequences. Chronic inflammation plays a significant role in the mediation of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, acquired immunodeficiency syndrome (AIDS), and dementia complex [77].

Natural pigments derived from microalgae (NPs) have neuroprotective properties, being valuable sources as functional ingredients in foods and pharmaceutical products that show efficient action in the treatment and/or prevention of neurodegenerative diseases. Vitamin E has preventive effects for many diseases, such as atherosclerosis and heart disease, as well as neurodegenerative diseases, such as multiple sclerosis [77].

Carotenoids have great potential benefits to human health, including the treatment of degenerative diseases, such as macular degeneration and cataract development. These compounds act as antioxidants, reducing oxidative damage by ROS. Studies indicated that increased intake of phenols decreased the occurrence of degenerative diseases. Phenolic compounds from microalgae with the potential to fight free radicals have been reported [121].

Dunaliella salina is a natural source of β-carotene, which produced a reduced risk of cancer and degenerative diseases in humans. Lutein is effective against various diseases, including cataracts and macular degeneration, and in the early stages of atherosclerosis. Extracts of *Chlorella* sp. containing β-carotene and lutein significantly prevented the cognitive disability that accompanies Alzheimer's disease in rats. It was also reported that lutein extracted from *Chlorella* reduced the incidence of cancer. Likewise, carotenoids extracted from *Chlorella ellipsoidea* and *Chlorella vulgaris* inhibited the growth of colon cancer [122]. A lycopene extracted from the microalgae *Chlorella marina* significantly reduced the proliferation of prostate cancer in mice [123]. This compound also reduced total cholesterol and low-density lipoprotein (LDL) levels [123] and improved rheumatoid arthritis [124].

Low plasma levels of lutein have also been associated with an increased tendency of myocardial infarction, whereas high intake of lutein was related to a decreased risk of stroke. In addition, high levels of carotenoids with provitamin A activity, including α-carotene, β-carotene, and β-cryptoxanthin, have been associated with reduction in the risk of angina pectoris. Macular degeneration, the leading cause of irreversible vision loss, has also been associated with very low consumption of lutein and zeaxanthin [125].

Scientific findings indicate astaxanthin for multimodal intervention for many forms of degenerative diseases, including cardiovascular diseases, cancer, metabolic syndrome, cognitive impairment, age-related immune dysfunction, stomach and ocular diseases (macular degeneration, cataract, glaucoma, diabetic retinopathy, and retinitis pigmentosa), and skin damage [126]. High levels of lycopene in plasma and tissues were inversely related to coronary heart disease, myocardial infarction, and the risk of atherosclerosis [125].

5.5. Compounds with Health Promoting Function. The importance of microalgae as sources of functional ingredients has been recognized because of their beneficial health effects. Natural pigments are valuable sources of bioactive compounds. These pigments have various beneficial biological activities such as antioxidant, anticancer, anti-inflammatory, antiobesity, antiangiogenic, and neuroprotective action and

are indicated for the treatment or prevention of several chronic diseases [77].

The antioxidant potential of carotenoid pigments and their ability to prevent cancer, aging, atherosclerosis, coronary heart disease, and degenerative diseases have been described. β-Carotene has higher provitamin A activity, which is essential for vision and the correct functioning of the immune system. Astaxanthin is linked to many health benefits such as protection against lipid peroxidation, age-related macular degeneration, reduced atherosclerosis, and an increased immune response [102].

Fucoxanthin is considered as a promising dietary and weight loss supplement and for the treatment of obesity. Clinical studies by Abidov et al. [127] demonstrated the effect of "xanthigen," a fucoxanthin based antiobesity supplement. Furthermore, fucoxanthin may be useful for the prevention of bone diseases such as osteoporosis and rheumatoid arthritis. It has also been reported to be effective for the therapeutic treatment of diabetic diseases, suppressing insulin and hyperglycemia [77].

Microalgae proteins are of great interest as a source of bioactive peptides due to their therapeutic potential in the treatment of various diseases [7]. Proteins, peptides, and amino acids have functions that contribute to health benefits. These compounds can include growth factors, hormones, and immunomodulators and can help to replace damaged tissues, in addition to providing nutritional benefits. Microalgae, such as *Chlorella* and *Spirulina* (*Arthrospira*), may be used as nutraceuticals or included in functional foods to prevent diseases and damage to cells and tissues due to their rich protein content and amino acid profile [102].

The antimicrobial action of certain enzymes (e.g., lysozyme) and immunoglobulins has been reported and recommended for people with different diseases (e.g., Crohn's disease) due to the existence of formulations with peptides and free amino acids. Studies of the health effects of lysozyme have been reported in the microalgae *Spirulina platensis* [128], *Chlorella* [129], and *Dunaliella salina* [130]. *Spirulina* (*Arthrospira*) and *Chlorella* biomass pills are marketed, as is "Hawaiian Spirulina Pacifica" (http://spirulina.greennutritionals.com.au/). Other proteins can also increase the production of cholecystokinin involved in appetite suppression and the reduction of LDL-cholesterol. Protein peptides from *Chlorella* have a potential as dietary supplements for the prevention of oxidative stress-related diseases, such as atherosclerosis, coronary heart disease, and cancer [39].

The essential fatty acids, ω-3 and ω-6 in particular, are important for the integrity of tissues. γ-Linolenic acid has therapeutic applications in cosmetics, to revitalize the skin and thus slow aging. Linoleic and linolenic acids are essential nutrients for the immune system and other related tissue regeneration processes. Linoleic acid is also used for the treatment of hyperplasia of the skin [102].

The most studied microalgal lipid compounds are the polyunsaturated fatty acids (PUFAs) docosahexaenoic acid (ω-3 C22:6) (DHA), eicosapentaenoic acid (C20 ω-3:5) (EPA), and arachidonic acid (ω-6 C20:4) (ARA). Studies have shown that dietary ω-3 PUFAs have a protective effect against atherosclerotic heart disease [131]. DHA and EPA showed the ability to reduce problems associated with strokes and arthritis, besides reducing hypertension, lipid content (a decrease in triglycerides and an increase of HDL) and acting as anti-inflammatory agents. DHA is also important in the development and function of the nervous system. Furthermore, ARA and EPA are platelet aggregators, vasoconstrictors, and vasodilators and have antiaggregative action on the endothelium, as well as chemostatic activity in neutrophils [102].

Other lipid compounds with interesting bioactive properties are the microalgal sterols. Phytosterols have demonstrated reduction of total cholesterol (LDL) in humans by inhibiting its absorption from the intestine [50]. Polysaccharides can be considered as dietary fibers associated with different physiological effects. Insoluble fiber (cellulose, hemicellulose, and lignin) mainly promotes the movement of material through the digestive system, thereby improving laxation and increasing satiety. They can also be considered as prebiotics because they promote the growth of gut microflora, including probiotic species. Soluble fiber (oligosaccharides, pectins, and β-glucans) may reduce cholesterol and regulate blood glucose [7, 132].

6. Conclusion

The proven ability of microalgae to produce bioactive compounds places these microorganisms in the biotechnological spotlight for applications in various areas of study, especially in the life sciences. The production of microalgal metabolites, which stimulate defense mechanisms in the human body, has spurred intense study of the application of microalgal biomass in various foods and pharmacological and medical products. There is obviously a need for further study of the identified compounds and their activities in the treatment and prevention of various diseases, in addition to an ongoing search for other, as yet undetected, metabolites.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Violacein: Properties and Production of a Versatile Bacterial Pigment

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Violacein-producing bacteria, with their striking purple hues, have undoubtedly piqued the curiosity of scientists since their first discovery. The bisindole violacein is formed by the condensation of two tryptophan molecules through the action of five proteins. The genes required for its production, *vioABCDE*, and the regulatory mechanisms employed have been studied within a small number of violacein-producing strains. As a compound, violacein is known to have diverse biological activities, including being an anticancer agent and being an antibiotic against *Staphylococcus aureus* and other Gram-positive pathogens. Identifying the biological roles of this pigmented molecule is of particular interest, and understanding violacein's function and mechanism of action has relevance to those unmasking any of its commercial or therapeutic benefits. Unfortunately, the production of violacein and its related derivatives is not easy and so various groups are also seeking to improve the fermentative yields of violacein through genetic engineering and synthetic biology. This review discusses the recent trends in the research and production of violacein by both natural and genetically modified bacterial strains.

1. Natural Violacein-Producing Strains and Their Locales Are Quite Diverse

As a bisindole, violacein (Figure 1) is produced by diverse genera of bacterial strains, including *Collimonas* [1], *Duganella* [2], *Janthinobacterium* [3–5], *Microbulbifer* sp. [6], and *Pseudoalteromonas* [7–9]. These violacein producers are varied phylogenetically and so are the locales from which they have been isolated. As shown in Table 1, these include quite a selection of environs as these bacteria have been found associated with the surfaces of sea sponges [7] and the rhizosphere of olive groves [10] and even within glaciers [4, 11, 12]. Perhaps the best known genus, however, is *Chromobacterium* [13, 14], which includes the strain *C. violaceum* [15].

2. Violacein as an Indicator of Quorum Sensing

In most of violacein-producing bacterial strains isolated from nature, this bisindole is a secondary metabolite that is associated with biofilm production [5]. Moreover, its production within *C. violaceum* and other strains is regulated by quorum sensing mechanisms [16]. Because it is easy to visualize, violacein production by *C. violaceum* has become a useful indicator of quorum sensing molecules and their inhibitors [17–20].

Secondary metabolites often serve functions other than the bacteria's immediate needs in growth and propagation. Many of these molecules are biologically active, and some have toxic properties to competing species giving the bacteria

Table 1: Some violacein-producing strains and the locales from which they were isolated.

Strain	Locale	Reference
Chromobacterium violaceum	River Waste water treatment plant	[14] [21]
Collimonas sp.	Arctic coastal waters	[1]
Duganella sp.	Agricultural soils (olive)	[10]
D. violaceinigra	Forest soils	[22]
Janthinobacterium lividum	Glacier	[4]
J. svalbardensis	Glacier	[11]
Pseudoalteromonas sp.	Deep sea waters (320 m)	[23]
P. luteoviolacea	Sea sponge surface	[7]

a competitive advantage. Because of these activities, many secondary metabolites have been found to have pharmacological properties and, thus, are of interest for clinical use. In the first half of this review, therefore, we will review some of the biological functions, clinical significance, and mechanisms of action of violacein.

3. Violacein's Function in Nature-Predator Defense?

As noted above, violacein-producing bacteria encompass various genera and are found in all types of natural environments, from marine to freshwater and soil environments (Table 1). For this reason, it is rather difficult to pinpoint violacein's main biological or ecological role. One possible commonality is that violacein producers are generally sessile bacteria which makes them more vulnerable to predation [6]. This leads to the idea that violacein serves as some sort of defense mechanism for its producing strain or that it provides these sessile bacteria some competitive advantage.

Consistent with this, violacein was shown to have antibacterial properties particularly towards Gram-positive bacterial strains [36, 37]. One strain in particular that has received most of the recent attention is *Staphylococcus aureus*, in part due to its status as a multidrug resistant pathogen [37–40]. Two studies showed that crude violacein was capable of inhibiting *S. aureus* growth at concentrations between 5.7 and 15 mg/L, or approximately 17 to 43 μmol/L [38, 40].

Violacein's biological activity is not limited to prokaryotes, however, as it is also known to have negative effects on bacteriovorus protozoans and metazoans (Table 2). Matz et al. tested purified violacein and violacein-producing biofilms with various protozoan species that include flagellates, ciliates, and amoeba [6]. They showed that, when exposed to violacein, the bacteriovorus amoeba *Acanthamoeba castellanii* displays decreased feeding accompanied by morphological changes, such as cell rounding. Further observations, such as increases in caspase-3-like activity and TUNEL assay studies, suggest that these predacious microbes are dying via an apoptotic-like cell death [41].

Like many protists, the soil nematode *C. elegans* is a bacteriovorus predator that ingests a wide variety of microorganisms [42]. Although *C. elegans* is best known as a premier genetic model organism that has propelled such important discoveries as RNAi, microRNAs, and programmed cell death [43–45], it is also an excellent tool to understand violacein's biological activity on bacterial predators. For example, *C. elegans* fed *E. coli* as a prey strain grows normally and healthily in the laboratory. However, two strains of bacteria that produce violacein, *Janthinobacterium* sp. HH01 and *C. violaceum,* were shown to be toxic to *C. elegans* [27, 46]. In particular HH01 exposures resulted in developmental problems in juvenile worms, as well as behavioral changes and a rapid mortality in adult worms. Interestingly, an exposure to a mutant HH01 strain that lacks violacein and tryptophan production did not induce severe toxic effects, indicating that the responses originated with the bisindole. As further confirmation of this, *E. coli* carrying HH01 vioA-E genes that produce violacein resulted in accelerated death, albeit at a slower rate than in HH01. These experiments affirm that violacein is likely toxic to the bacterial predator *C. elegans*.

In addition to its apparent roles in protecting the bacterium from predation, violacein seems to have other ecological functions that may provide advantages for the bacteria that produce it. For instance, violacein-producing *Janthinobacterium* forms biofilms and resides on the skins of frogs and salamanders [24, 47]. In return, the violacein produced provides antifungal protection for its amphibian hosts, increasing their survival.

4. Potential Clinical Uses of Violacein

These functional studies portray the secondary metabolite violacein as a toxic sentinel guarding against diverse potential bacterial predators and other competitors. However, such a broad cellular toxicity may also prove to be useful as a therapeutic against various pathogenic and endogenous cellular insults. Consistent with this, many pharmacological properties have been attributed to violacein.

Violacein has strong antibacterial effects making it a promising candidate as an antibiotic. Moreover, when administered in combination with other antibiotics, the impact is more effective in fighting bacteria than the use of antibiotics alone [48]. This is of particular interest in light of recent antibiotic-resistant strains of pathogenic bacteria, such as MRSA. Also, the antiprotozoan properties of violacein could be exploited to treat diseases in humans, such as in malaria and leishmaniasis [25, 26].

The most studied clinical use of violacein, however, is it being a potential cancer therapeutic. Violacein has been tested against various cancer cell lines (Table 3), where it has shown cytotoxicity at IC50 values that mostly range in the submicromolar concentrations. The effects of violacein were also shown to be specific for the cancer cell line tested as two colorectal cancer cell lines, Caco-2 and HT-29, were differentially susceptible to violacein [31]. Since violacein is cytotoxic towards noncancer cells as well [28, 34], the critical

Table 2: Eukaryotic organisms in which violacein was shown to produce negative effects.

Type	Organism	Description	Reference
Fungi	*Batrachochytrium dendrobatidis*	Infects amphibians	[24]
Protozoa	*Rhynchomonas nasuta*	Flagellate	[6]
	Tetrahymena sp.	Ciliate	[6]
	Acanthamoeba castellanii	Amoeba	[6]
	Leishmania amazonensis	Causative agent of leishmaniasis	[25]
	Plasmodium falciparum	Causative agent of malaria in humans	[26]
	Plasmodium chabaudi chabaudi	Causative agent of malaria in mice	[26]
Nematode	*Caenorhabditis elegans*		[27]

Table 3: List of cell lines tested against violacein.

Cell line	Cell type	Organism	Notes	Reference
V79	Fibroblast-like cell line from lung tissue	Chinese Hamster		[28]
FRhK-4	Fetal kidney	Monkey		[29]
Vero	Kidney	Monkey		[29]
MA104	Kidney epithelial cells	Monkey		[29]
Hep2	Hela-derived	Human		[29]
92.1	Uveal melanoma	Human		[30]
OCM-1	Choroidal melanoma	Human		[30]
NCI-H460	Non-small-cell lung cancer	Human		[28]
KM12	Colon cancer	Human		[28]
Caco-2	Heterogeneous epithelial colorectal adenocarcinoma	Human		[31, 32]
HT29	Colorectal adenocarcinoma	Human		[31]
HCT116	Colorectal adenocarcinoma	Human		[32]
SW480	Colorectal adenocarcinoma	Human		[32]
DLD1	Colorectal adenocarcinoma	Human		[32]
TF1	Erythroleukemia	Human		[33]
K562	Lymphoma	Human	N/C[a]	[34]
U937	Chronic myelogenic leukemia	Human	N/C[a]	[34]
HL60	Promyelocytic leukemia	Human		[34]
MOLT-4	Acute lymphoblastic leukemia	Human		[28]
EAT	Ehrlich ascites tumor	Mouse	*In vivo*[b]	[35]

[a]No cytotoxicity observed.
[b]Both *in vitro* and *in vivo* tests were performed.

factor for its clinical use against cancer is that it is more toxic to cancer cells than to normal cells. One study demonstrated that violacein induced apoptosis in HL60 cells (IC50 = 700 nM), a cancer cell line used as a model to study myeloid leukemia. However, normal lymphocytes were unaffected at the concentrations tested, further asserting violacein's use as a putative cancer therapeutic [34].

Violacein clearly has toxic effects on cultured cancer cells, that is, within *in vitro* tests. Its ability to attenuate cancer growth *in vivo* was also tested recently in the Ehrlich ascites tumor (EAT) mouse model [35]. Daily intraperitoneal injections of 0.1 μg/kg violacein significantly increased the survival rate of the mice, while no adverse effects were observed in mice receiving higher doses of up to 1 mg/kg.

5. Biological Mechanism of Violacein's Effects

Even though violacein is a promising agent as an antibiotic and a treatment for cancer, the biological mechanisms behind these actions remain elusive. Before we can stamp violacein as a natural therapeutic for numerous kinds of diseases, it is imperative to understand its mechanisms of action at the cellular and molecular levels.

Several groups have begun this and many of the studies performed in cancer cell lines report increased activity in indicators of apoptosis-related markers, such as increases in the reactive oxygen species (ROS) and the activation of caspases [31, 32, 34, 35]. However not all cancer cell lines respond to violacein and the reason for the selectivity is not

well understood. Moreover, among the leukemia cell lines tested in the literature, violacein showed selective cytotoxicity against HL60 and TF1 (Table 3), but the pathways that lead to cell death were very different in the two cells. In HL60 cells, an exposure to violacein led to phosphorylation of p38 MAP kinase, upregulation of the NFκB pathway, and activation of caspases [34]. It was also found that TGFα receptor activation was required for these downstream effects. TF1 cells, on the other hand, did not seem to follow the canonical apoptotic pathway as treatment with inhibitors of proapoptotic caspases did not prevent cell death [33].

Conclusions on violacein's mechanism of action are clearly scant at this point. However, the fact that violacein has cytotoxic effects on such a wide variety of organisms and cells hints at a common target or pathway. Studying the effect of these bisindoles at the genetic level on model eukaryotic organisms, such as *C. elegans*, will help in elucidating its mechanism of action and enrich our knowledge of violacein as a clinical therapeutic. Owing to its versatile activity against many human ailments and infectious agents, however, it is not surprising that this bisindole has garnered more attention recently from the scientific community. One factor that may contribute to reducing violacein's application, though, is its relatively low level production by natural strains. Consequently, the latter half of this review will be primarily given to the discussion of current research into the production and purification of violacein and its related derivatives.

6. Production of Violacein by Natural Host Strains

Since violacein is produced naturally by various bacterial species, the use of these strains for its production seemed like a clear choice. However, many factors were found to influence the yields, including the agitation and aeration [7], the inoculum size [2, 10], and the nutrients available [2, 10, 49, 50]. It should be noted that the violacein concentrations reported within many of the articles are based upon the extinction coefficient as determined by the authors using spectrophotometric analyses, with extinction coefficient values ranging between 10.955 and 74.3 L/(g-cm) in the literature [2, 49, 51, 52]. A recent article by Rodrigues et al. highlighted the discrepancy caused by spectrophotometric-based determinations of violacein and deoxyviolacein concentrations and stated that this could result in violacein concentrations that are inflated by as much as 680% [51]. To address this in their study, therefore, Rettori and Durán (1998) relied on HPLC measurements, a technique which was proposed in an earlier study to be used in parallel with NMR, UV-Vis spectroscopy, and mass spectroscopy when characterizing violacein and its production by bacterial strains [52]. Consequently, to avoid any potential confusion to the readers, this report will provide the violacein and deoxyviolacein concentrations reported and state whether they were determined via HPLC or with an extinction coefficient.

One group applied response surface methodologies (RSM) to identify the best conditions to produce violacein with *C. violaceum* [49]. They initially analyzed 16 variables but eventually limited them to three—glucose, tryptone, and yeast extract. While the latter two improved both the cell mass and violacein yields as they were increased, glucose was found to be negatively correlated with the violacein yields, and limiting its addition to the culture was advantageous. Using this technique, they were able to increase the dry cell weight (DCW) from 7.5 to 21 g/L and the violacein yields from 170 to 430 mg/L with an extinction coefficient of 56.01 L/(g-cm). Although there was a significant improvement in the volumetric productivity of violacein, defined as the mg of product per liter of culture, it should be noted that this does not represent a greater specific productivity of this bisindole, that is, mg product per gram of cells, as the cell mass increased by 2.8-fold while the violacein concentration increased by only 2.5-fold. Yet, similar protocols could potentially be employed with other strains to increase the cell density and, thus, raise the volumetric productivity.

A more recent study using RSM with another violacein-producing strain, *Pseudoduganella* sp. B2, previously *Duganella* sp. B2, found similar results as the concentration of beef extract used was a major impetus for violacein production [2]. They also identified the culture pH and the concentrations of tryptophan and potassium nitrate as major players influencing the final violacein yields. The importance of tryptophan is not surprising as this is the precursor for violacein, while the impact of nitrate was thought to be related to nitrogen source availability for the growing bacterial cultures. Under the optimal conditions found, the authors claimed *Duganella* sp. B2 was capable of producing 1.6 g/L crude violacein, typically referring to the naturally produced mixture of violacein and deoxyviolacein. This value is under scrutiny, however, as the extinction coefficient used (10.955 L/(g-cm)) is the lowest reported in the literature [2, 49, 51, 52].

7. Production of Violacein within *E. coli* and Other Heterologous Hosts

Since the genes required for the production of violacein are known to exist within a single operon, that is, *vioABCDE* [53], many groups have sought to clone and express these within other bacterial hosts, including *E. coli* [8, 51, 53]. The Pemberton group has focused on plasmid stability, an issue when trying to generate bacterial products in long term and in nonnatural hosts. They found, for instance, when the violacein gene cluster was cloned into pHC79, a cosmid vector, that it was unstable and was lost in as much as 60% of the bacterial population when grown for 15 generations in the absence of antibiotic pressure [54]. They claimed that the same was true when they expressed the *vioABCDE* operon in a pUC18 vector but were able to generate a stable construct using a broad host range IncP plasmid [54, 55]. This plasmid, pPSX-Vio$^+$, was stable without antibiotics for more than 100 generations [54], making it a potentially useful tool for the production of violacein.

In a subsequent study, they found that the amount of violacein produced by *E. coli* was dependent upon the host, with *E. coli* strain JM109 producing 3.9-fold more violacein than *E. coli* strain DH5α when harboring the same plasmid [55]. The production of the alpha amylase protein, AmyA, from *Streptomyces lividans* was likewise found to be better in

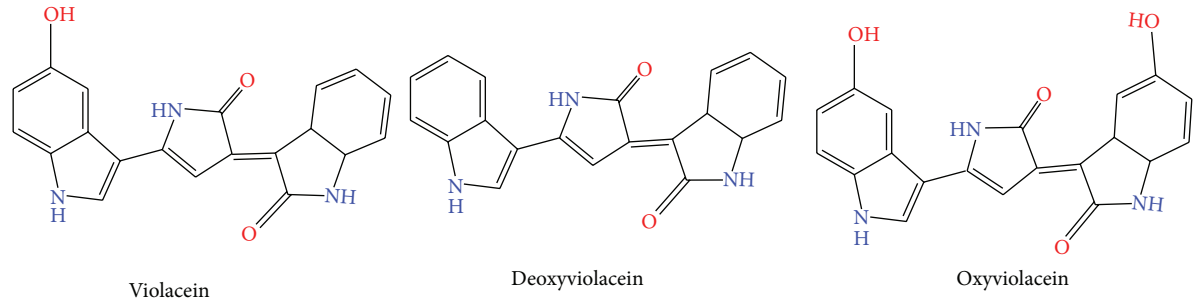

Figure 1: Structures of violacein, deoxyviolacein, and oxyviolacein showing either the presence or the lack of the hydroxyl groups. The structures were prepared using ChemDoodle 2D sketcher (http://web.chemdoodle.com/demos/sketcher).

JM109 than DH5α, demonstrating that this phenotype was characteristic of JM109 and not due to the violacein genes. The authors attributed better results with JM109 to the genetic differences between the host strains but did not delve deeper into the mechanisms underlying this phenotype. However, they did identify a mutation within the plasmid which led to a further enhancement in the violacein yields [55]. This mutation, which the authors designated as *opv*-1 (overproduction of violacein), results from the deletion of a single nucleotide within the region upstream of the *vioA* gene, leading to a 4.2- and 2.9-fold enhanced violacein yield from *E. coli* strains DH5α and JM109, respectively.

A more recent study sought to engineer *E. coli and* its metabolic pathways to improve the violacein yields [51]. For this, the authors overexpressed the genes related to tryptophan production and knocked out several genes and pathways which would detract the carbon flux away from this amino acid. The engineered *E. coli* strain, TRP11, produces about 20 μmol tryptophan per gram DCW (gDCW). By comparison, the control wild-type strain only produced about 0.3 μmol tryptophan/gDCW, representing an increase in the tryptophan concentration of more the 60-fold. They next introduced the *vioD* gene into the chromosome of this strain and a plasmid expressing the *vioABCE* genes. Performing fed-batch fermentations over a 12-day period with this strain, which they designated as Vio-4, they were able to generate 710 mg/L violacein at more than 99% purity, demonstrating that *E. coli* can be used to produce high level concentrations of this bisindole specifically. *E. coli*, however, has not been the only strain to have been used as a host to produce the bisindole violacein. Both *Citrobacter freundii* and *Enterobacter aerogenes* have also been used with positive results [56].

8. Deoxyviolacein and Oxyviolacein

Although violacein has received most of the attention, it is not the only compound being produced by the VioA, VioB, VioC, VioD, and VioE proteins within these bacterial hosts. A recent article that describes the various products in detail was recently published and lists over a dozen different compounds that have been produced in tests with various strains, mutants, and cell-free extracts [13]. Perhaps the best known derivative produced by the violacein biosynthetic pathway, however, is deoxyviolacein. In *J. lividum*, for example, deoxyviolacein (Figure 1) is also being produced, albeit at a lower level than violacein [57]. Similar results were also seen with *Duganella* sp. B2 where the production of deoxyviolacein was lower than that of violacein, as measured by HPLC [58]. The percent deoxyviolacein within the crude violacein extracts obtained from the natural bacterial hosts is typically around 10~20%, with the vast majority being violacein. This is also true for commercially available violacein from Sigma-Aldrich, which is prepared using *J. lividum* and certified as at least 85% violacein based upon HPLC analysis.

Deoxyviolacein, which lacks a hydroxyl group (Figure 1), can be produced by omitting the VioD protein. Using a recombinant *Citrobacter freundii* carrying a plasmid with a knock-out in the *vioD* gene, a recent study by the Xing group reported on the high level production and characterization of deoxyviolacein [58], with an emphasis given to the differences between violacein and deoxyviolacein. Their study showed, for example, that the photostability of deoxyviolacein was slightly better than violacein in tests with either natural or UV light. Moreover, in 24-hour toxicity tests with HepG2 cell lines, violacein and deoxyviolacein were both found to be toxic. However, the impact of violacein was dose-dependent as greater additions of this bisindole led to greater concomitant losses in the HepG2 viability. In contrast, the toxicity of deoxyviolacein was not dose-dependent but led to fairly consistent and stable losses in the viability over a range of concentrations (0.1–10 μM). The difference between these two bisindoles was even more pronounced when the viability was determined after 48 hours.

Several groups have also shown that the proteins responsible for the biosynthesis of violacein are not strict for their typical substrate, tryptophan, as 5-hydroxytryptophan can also be used to produce another derivative, oxyviolacein (Figure 1) [59, 60]. In contrast to deoxyviolacein which lacks a hydroxyl group when compared with violacein, oxyviolacein boasts an additional hydroxyl group. Likewise, as the loss of the hydroxyl group in deoxyviolacein reduced its efficacy against *S. aureus*, the presence of this extra hydroxyl group within oxyviolacein was found to increase the potency against these human pathogens [61].

9. Conclusions

This review presents current research trends regarding the study and production of the bacterially produced bisindole violacein and several derivatives. As a secondary metabolite,

violacein has been found to possess a wide variety of biological activities, including anticancerous properties. These characteristics have led to renewed interest in this compound and its production by both wild-type and recombinant bacterial strains. As presented in this report, the production and characterization of violacein are not without their own obstacles and struggles, and much work still needs to be done. This is particularly true regarding the mode of activity of violacein which needs to be studied more in depth. Current trends in molecular genetics are aiding in this as researchers are now capable of engineering bacterial host that can overproduce this bisindole within fermentations. As work with this compound and its derivatives progresses, it is anticipated that violacein will become more readily available for the scientific community and clinical studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Seong Yeol Choi and Kyoung-hye Yoon contributed equally to this paper. All authors participated in preparation of this paper.

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