Bioactivity evaluation of *Streptomyces* Pigments from Arauca and Guaviare rivers with potential in the cosmetic Industry.

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Bioactivity evaluation of *Streptomyces* Pigments from Arauca and Guaviare rivers with potential in the cosmetic Industry.

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Acknowledgments

The following research project has been the work of two and a half years. Two years of many adventures, anecdotes, and, most importantly, infinite learning. I want to dedicate a few pages to the people who will have the possibility of reading this document, to those who accompanied me on this journey, to those who are no longer here, and to all those who will read it as a contribution to their future research.

Studying for a master's degree implies many structural changes in your life. It is a moment of deconstruction of what you have already learned. It is unlearning and learning in unknown territory. It is to broaden your world with another perspective; it is to prepare yourself not only for the working life you dream of but also for your personal life. The professional you want to be will transform the human being you are today. Alice Walker says, look closely at the present you are building; it should look like the future you are dreaming of.

In 2019, a dream began, an illusion that has always motivated me since I was a child, those who have known me since childhood has witnessed my curiosity, persistence, and absolute love for science. Today I culminate a stage in my life that, in a way, made me see many facets of myself that I did not know. Additionally, fortunately, I see it this way today; I had to complete it at a time when uncertainty, anxiety, nerves, loneliness, and many emotions that made us feel like a roller coaster would be the new emotional state of the world for the next 24 months. COVID is the moment before and after for everyone living in the 21st century. A pandemic that we never imagined living through, that was distant to us, or only observed from a television set or story books.

If God and life grant me the privilege of having children, one of the anecdotes that I will tell you that has most marked my life will be this one.

Today I look back at myself two years ago and now. I remember many of the questions, phrases, and comments I received, "How much do you want to study? You could invest what your master's degree costs in a business.
Are you sure? You already did a master's degree; will you do another one? My friends and family often questioned me, and when I would get mad, many of their phrases would come to my head, I told you so! You had an example, but you wanted to. You must finish what you start, remember that, let it be a Law of Life. It is true; what happens to people who want to study for a master's degree? This question should be a thesis in philosophy. Maybe I don't have the right words to answer a question as complex as this one. Still, I can argue from what I have learned to aspire to a master's degree, and maybe that will answer, in some way or another, why the crazy people in love with the strangest things in the world get the idea to do a master's degree.

My adventure on this path of science only left me with lessons that will remain in my memory, which is a more than practical reason why I should pursue a master's degree. Many people will say, Lau, you are crazy, and I will answer with a resounding yes. What

would the world be without people with inquiring minds? For me, the madness of learning is a carousel of parasympathetic sensations that activate my whole brain and body, make my mind dance, and exalt my heart with energy. Today I consider myself a knowledge replicator; today, I consider myself the best marketer of *Streptomyces* and, in fact, maybe the only one. I bet no one posts pictures on their Instagram of their microbial creation smelling like dirt and strange aromas from a microbiology lab. Hi, I am Laura Dexter, and I am deeply in love with my *Streptomyces*. And this is what is truly unique; I have no scientific explanation for it; maybe it exists, it would be worth studying, and I would surely be part of the group of volunteers, but I must confess that the connection that was generated between me and my bacteria belongs to another world. My colleague, the professor, told me, Lau, the holy spirit is with you. Gratitude, pure and good affection, is what my little critters gave me, my affectionate way of referring to them. I don't know if my serenades from 4-11 at night, my words full of positivism every time I saw them, or my dance rituals every time I put them in chaotic agitation so that they would grow for days. It is a reality that loves is universal and full of mystery. Love has no definition; it is complex; it is chemistry, it is physics, it is biology, it is a contradiction, a fascination, sometimes it inspires us, sometimes it hurts us. As Gandhi says, where there is love, there is life. And this, readers, is Science. This means to be Magister, a big reason to study and see the world through a Petri dish.

Today I see what, many times was tiredness, exhaustion, frustration, and desire not to try anymore as a way that life told me, Laura, you must live! In the middle of a pandemic, with a small heart, broken, nostalgia, and melancholy. My bacteria marked a milestone; they reconciled me with love, life, and my inner child. They lifted my spirits; their bright color injected me with vitamins and gave me the strength and resilience that research implies. This is reflected in their results and how proud I feel of myself as a person and as a professional. All of us who dedicate our lives to science rejoice our souls to see that our efforts, despite the tantrums, arguments, and other not-so-pleasant but necessary emotions, lead us to things, moments, and discoveries that transcend and contribute to creating knowledge societies.

Today, July 15, I deliver my degree work, which has been elaborated with love, euphoria, and many moments of frustration, sadness, and anger. A cocktail of feelings that have been the perfect ingredient to complete one more goal in my life.

I want to begin my thanks to the most important people in my life. Mainly to my family, who have been my engine, my teaching, and my guide in my 28 years of youth, and of course, my support in my moments of weakness and not wanting to continue resisting and persisting.

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Today I close a chapter of my life, but I open another one to continue writing more stories, cultivating my curiosity, and never stop learning.

I wanted to make these acknowledgments uniquely; I tried to summarize in these five pages what this journey through the world of science meant. Today, I consider myself more ignorant; there is a whole world to discover. Maybe I will never finish finding new things, but I am sure that I will never stop being surprised. I will continue to cultivate this thirst for constant learning. I will continue to sharpen my critical ability. My sense of curiosity. Today I join the movement of those who like to ask 'Why'? And finally, to thank again the people who will take the time to feel, to read and reread, to cry, to be moved, to remain silent, and keep these words that I dedicate to you with much love.

With love and a lot of butterflies fluttering in my stomach, being 12:45 pm on July 7, 2022, listening to Daft Punk's Instant Crush, I dedicate this work to

My parents
Sister
Grandfather
Family
Friends

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ABSTRACT

Personal care is one of the essential activities for human beings today. Cosmetics are mixtures of multiple ingredients, the purpose of which is to provide a benefit on the skin. This industry has a turnover of more than 429.8 million annually and a growing trend of 4.6%. This behavior has led to the inclusion of a new segment known as natural cosmetics, whose purpose is to promote the use of natural and more environmentally friendly ingredients. One of the most controversial ingredients in cosmetics is synthetic dyes and pigments. These compounds have been exposed in the scientific community with great concern for contributing to the generation of free radicals, their high content of heavy metals, and being precursors of hormonal disorders and other degenerative diseases. Consequently, the search for more eco-friendly and healthier pigment sources that are easy to handle and feasible in industrial production has been encouraged. One promising source of natural pigments is from microorganisms. The most representative genus of this type of secondary metabolite is *Streptomyces*. These actinomycetes are of great interest to the many biotechnological products currently marketed worldwide. This research aimed to evaluate the bioactivities (antimicrobial, antioxidant, cytotoxic, anti-inflammatory, tyrosinase inhibition, and anti-acne activity) of *Streptomyces* strains from Colombian rivers that showed coloration in culture and antifungal activity in other studies. Additionally, identify which variables can be optimized in the bioproduction of the pigment, and how its bioactivity behaves, to validate whether this pigment can be considered as a possible raw material for the cosmetic industry.

The results of this research are summarized as follow: twenty strains showed pigmentation in agar, of which only three (290, 145, 627 strains) maintained the pigment in a liquid ISP2 medium. The colored crude extracts were prepared in a mixture of water:ethanol (70/30 v/v), which were concentrated by rotaevaporation. Extracts were evaluated against 10 pathogenic strains using the disc diffusion technique. It was obtained that the colored extracts of 290, and 145 strains, presented antimicrobial activity against *B. subtilis*, *E. faecium*, *S. aureus*, and *S. epidermidis*. The colored extract (CE) of strain 627 showed no antimicrobial activity. Antioxidant activity was performed by DPPH (1, 1diphenyl-2-picryl hydrazyl) radical scavenging method for these extracts, at concentration of 10 mg/mL. They exhibited moderated antioxidant DPPH radical scavening activity (22.06%, 5.27%, 53.73% for 145, 290 and 627 strains respectively). The extracts from 290 and 145 strains presented potential cytotoxic activity by MTT (3-(4,5-Dimethylhiazol-2-yl)-2,5-diphenyltetrazolium bromide) against HeLa cells with an IC_{50} 93.21 and 37.34 μg/ml, respectively. The extract from 627 strain did not present cytotoxicity against HDFa cells. Colored extract from 145 strain was selected as the pigment with the best bioactive results, so it was subjected to fractionation with 100% ethyl acetate, and the antimicrobial activity was evaluated by a growth curve against *S. epidermidis*, whose growth rate was equal to that of the control Gentamicin® (10 μg), reporting 91% inhibition in a time of 18 hours. Determining variables that affect pigment production was conducted first, choosing the carbon and nitrogen source that will most affect pigment production in a solid medium. The result corresponded to soluble starch,

and in nitrogen source, no change was perceived. Six factors were evaluated based on a Plackett-Burman experimental design. The most influential factors were temperature, agitation, and incubation time. Run 1 corresponded to the highest production of crude pigment, with a concentration of 85.33 µg/mL. The optimization was based on these three positive variables, and a response surface analysis was performed; of the 15 runs, the following optimum conditions for crude pigment production were determined: 30, 50 rpm, and 7 days of incubation. FTIR analysis indicated the presence of C-C, C-N, C-O, C≡C, C≡O, and C≡N, in complement with LS/MS; the presence of three peaks was determined with the possibility that it could be a compound already reported. *Streptomyces* strain 145 corresponds to *Streptomyces parvulus*. The fractionated pigment of *Streptomyces parvulus* showed a 36.89% inhibition of TNF-α interleukins and a significant effect on IL-10 production.

As conclusions: *Streptomyces* pigments from Colombian rivers can be considered possible ingredients for the cosmetic industry since they meet at least 2 minimum bioactivities required of those evaluated. The fractionated pigment 145, can be postulated as a candidate for a cosmetic ingredient because it presented moderate antioxidant and cytotoxic activities; it is potentially antimicrobial against pathogenic strains that cause many skin diseases, as is the case of *S. epidermidis*. Additionally, it had a significant anti-inflammatory effect on lipopolysaccharide-treated macrophages against ibuprofen and presents a limited depigmenting activity that can be potentiated.

1 CHAPTER 1: INTRODUCTION

1.1 CONTEXT AND PROBLEM

Today, the trend of skincare, healthy eating, free of synthetic chemicals, toxic substances in pesticides and preservatives cause more and more people in the world to transform their consumption habits or to question everything the market offers[1], one of the most studied and controversial industrial applications is those of colorants, due to the extensive use of these in different industries such as food, textiles, pharmaceuticals, and cosmetics; Its main function is to exalt the product, make it more attractive in terms of marketing [1], [2], however, these substances are generating free radicals or have a high load on heavy metals, which are found or deposited in the products that are consumed or in the cosmetics that are applied, being the main precursors of metabolic disorders and genetically influencing the body to develop immunological diseases such as arthritis, cancer or accelerate premature aging of the body [1]. In addition, there is a high environmental impact that concerns the production of these substances. It is estimated that around 100,000 dyes and pigments are marketed today, which represents 700.000 tons of production, translating it between 10-15% of the effluent contamination [3]. This rate of both production and pollution is due to the growing demand of the colorants market worldwide, with projected sales by 2025 of approximately US$ 37.490 million in 2025 [4]. On the other hand, the category of natural colorants will present an increase of 7% for the period 2018-2022, equivalent to US$ 437.54 million dollars in sales [4]. In the case of Colombia, the market for natural colorants has grown 70.85% in the last years, developing more sustainable industries and with greater participation in the international markets, however, imports of synthetic colorants are still much higher than domestic production [5].

Natural pigments are an alternative to replace organic and inorganic pigments of synthetic origin; In general, they are obtained from seeds, plants, fruits, and roots and depend heavily on variables such as climate, soil types, cell tissue types, seasonality, among others, which makes current production methods economically unviable processes [2], for cosmetic and pharmaceutical sectors, significantly increasing the value of the product in the market and its availability. Accordingly, research has been carried out around the production of natural pigments from microorganisms, since the production process is more feasible in economic terms and pigment production does not depend on climatic, topographic or cellular conditions [2]. In comparison to the obtaining of pigments by plants, but of the manipulation of variables of culture, as it is the case of the Actinobacteria [6].

1.2 JUSTIFICATION

One of the most advanced research projects in the production of *Streptomyces* pigments is the obtention of melanin, nowadays under different fermentation processes (some patented) they have managed to scale it up industrially, and it is widely used in the development of pharmaceutical products and some sunscreens due to its high UV and antioxidant activity [7]–[9]. However, most *Streptomyces* pigments are evaluated for the food industry; from the literature review conducted for this research, very few studies point to cosmetic applications [10]. On the other hand, Colombia has important microbial biodiversity, given the microclimate conditions that favor the growth of microorganisms in different habitats [11], [12] . This allows the development of many investigations to obtain materials or ingredients that do not come from non-renewable sources or that deteriorate the ecosystem, and insofar as possible that do not generate a long-term environmental impact. Therefore, obtaining secondary metabolites or therapeutic agents from microorganisms, gives an economic and sustainable boost to the country [11].

In addition, Colombia is entering the development of organic and ecological products with a strong participation in the foreign market. For the year 2019, more than USD $486.300 were consolidated in exports of green products [13]. On the other hand, the country achieved a growth of 3. 9% in exports of natural ingredients and cosmetics by 2018; this is attributed to the global trend outlined by Euromonitor on changes in consumption and consumer preferences on ecological and sustainable products [14], therefore, are prioritizing resources and innovations to achieve the goal of the productivity program of the Ministry of Industry and Commerce, which is: By the year 2032, to turn Colombia into a leading country in ingredients and cosmetic products, of natural origin, therefore, to carry out the development of research based on this type of microorganisms can achieve advances in tangible materials, such as cosmeceutical ingredients, which can be used for the development of natural cosmetic products that revolutionize the industry, generate employment and contribute to the objective proposed by the ministry [11].

In addition, there is currently no established methodology that is standardized and appropriate for the production, extraction, and subsequent use of pigments obtained from *Streptomyces*. Therefore, this research aims to obtain *Streptomyces* pigments by establishing the best culture conditions for their bioproduction and to evaluate the bioactive potential of these pigments for their possible application in the cosmetic industry [15]–[17]. Following the above, the following research question arises: What are the main factors in *Streptomyces* culture that favor the bioproduction of bioactive pigments with possible cosmetic application?

1.3 OBJECTIVES

1.3.1 Main objective

To Establish the culture conditions for *Streptomyces* isolated from the Guaviare and Arauca rivers that favor the production of bioactive pigments.

1.3.2 Specific Objectives

- To evaluate the bioactivity (antioxidant, cytotoxic, antibacterial, tyrosinase inhibition, anti-inflammatory and anti-acne) of colored extracts from the fermentation of *Streptomyces*.

- To evaluate the effects of *Streptomyces* culture variables (Temperature, pH, carbon concentration, nitrogen concentration, incubation time, agitation velocity) in the production of bioactive pigments at the laboratory scale.

- Characterize the content of metabolites present in the enriched fraction of the colored extract using fine chemistry techniques (HPLC-L/MS and FTIR).

1.4 DOCUMENT OUTLINE

This paper presents the results of a master's research. In pursuit of the proposed objective, different approaches were carried out, which are explained in four chapters as follows:

- Chapter 2 contains a literature review and background of *Streptomyces* strains as potential bioactive pigment producers.

- Chapter 3 includes evaluating preliminary bioactivities of *Streptomyces* colored extracts to determine their potential as a possible cosmeceutical ingredient.

- Chapter 4 contains statistical models of factors with the most significant favorable influence on *Streptomyces* pigment production and their respective optimization.

- Chapter 5 consists of general conclusions and suggestions for future research.

2 CHAPTER 2: *STREPTOMYCES*-DERIVED BIOACTIVE PIGMENTS: A SYSTEMATIC LITERATURE REVIEW

2.1 ABSTRACT

Pigments are widely used compounds. They are practically part of many of man's daily activities. However, in recent years, concern has been raised regarding these compounds' human health and environmental impact. This is due to the type of pigment produced on a large scale. Currently, most pigments are of synthetic origin. These highly complex molecules are created with chemical substances and compounds that harm human health and nature. This has generated a search for new ways of obtaining pigments that are safer and friendlier to the planet earth. Therefore, much research has been directed to study pigments produced by microorganisms that are easier to adapt to any environment, manipulate genetically, and are also a renewable source of raw material. One of the most representative microorganisms in pigment production is *Streptomyces*. This genus of actinomycetes has a great biotechnological value since it contributes to most antibiotics marketed today, among other secondary metabolites of great interest. Here presented the results of a systematic literature review in which pigments produced by *Streptomyces* with bioactive potential were evaluated. Among the most outstanding bioactivities, antimicrobial, antioxidant, and cytotoxic effects were found. On the other hand, the factors or variables affecting pigment production were analyzed, finding the most common: temperature, pH, substrate sources, and incubation time. Additionally, statistical data were collected from scientific publications on this subject to understand the development of *Streptomyces* pigment production in recent years. These results indicate that *Streptomyces* are a source of pigments with potential application for the development of new products that are safe and more environmentally friendly.

Keywords: *Streptomyces*, Pigments, Antioxidant, Antimicrobial, Cytotoxic,

2.2 INTRODUCTION

Pigments are used in the manufacture of various products because they can enhance the natural color or replace the color that has been lost during the manufacturing process, generating greater consumer appeal by adding a novel sensory aspect. Since the introduction of synthetic dyes by Perkin in 1856 [18], their production has increased, and natural colorants from plants and animals have decreased due to synthetic pigments being relatively cheaper [19]. In the 20th century, natural organic pigments were almost entirely displaced by synthetic molecules such as phthalocyanines ranging from blue to green and quinacridones ranging from orange to violet [20]. Advances in organic chemistry allowed these compounds' mass production to replace the natural ones, which are often more complex to acquire [21]. Therefore, synthetic organic dyes have been the most cost-effective approach for years [22]. Synthetic dyes are superior to natural pigments in their staining power, ease of application, stability, and cost/effect [23], [24]. However, from the point of view of health safety, consumers do not accept them as they have been associated with carcinogenesis and teratogenesis [25]; due to this, there has been a growing interest in recent years in natural dyes, mainly in the food and cosmetic industry [26], [27].

Since 1975 the FDA (Food and Drug Administration) has conducted toxicological studies on synthetic food dyes, finding different irregularities, namely the lack of statistical reliability of the number of animals used and inadequate dosing [28]. It has also been associated with cancer in experimental animals, where dyes such as red 40 or Allura (listed as permanently approved by the FDA) promote tumor formation [29]. These controversies between synthetic dyes and pigments have contributed to the worldwide trend of replacing synthetic dyes with natural pigments. The pigments extracted from plants or microorganisms imply a certain degree of safety. Due to historical antecedents and consumption patterns, toxicological problems are not as marked as their synthetic counterparts [30].

The use of natural pigments in food, paints, cosmetics, and pharmaceuticals has increased in recent decades [31]. Natural pigments can be obtained from three primary sources: animals, plants, and microorganisms [32]. Although there are many natural pigments, only a few are available in quantities suitable for industrial production[33], [34]. Some bacteria produce pigments, which can be observed after growth in colonies, which are helpful for colony identification [35]. The most used natural pigments in the industry are riboflavins extracted from Bacillus subtilis and used in the food industry, vitamin-enriched dairy products, and energy drinks [18]. Microbial pigments are of great interest due to their stability and technological availability [36], [37]. The benefits of pigment production from microorganisms include easy and fast growth in economic culture media, independence from climatic conditions, and different colors and shades [38], [39]. Thus, microbial pigment production is now one of the promising and emerging fields of research, revealing its potential for various industrial applications [32]–[34], [40], [41]. Additionally, some microbial pigments have been reported to possess anti-cancer activity, contain pro-vitamin A and have some essential properties such as stability to light, heat, and pH [42]. However, from an industrial point of view, developing a high-tech and cost-effective

harnessing for large-scale production of various microbial pigments is necessary [34]. Among microbial pigments, one of the most exciting genera is *Streptomyces* due to its great reproductive capacity. Melanin is one of the most produced pigments in the industry from this bacterium. In addition, this type of actinomycetes has a fascinating genetic distribution, which is attractive for replication in the biotechnology industry [43]–[45]. In addition, *Streptomyces* are well-known for their abundant secondary metabolism, which has provided different bioactive compounds, namely antibiotics, anti-inflammatory, antioxidant, and cytotoxic [45]–[48]. Several of these compounds are colored [34], and, given the bioactivity potential shown for *Streptomyces* strains [49], many-colored *Streptomyces*-derived compounds could signify an exciting opportunity to find bioactive pigments.

Considering the need for safe pigments applicable in different areas with additional beneficial properties and taking advantage of the biotechnological potential of *Streptomyces*. The following systematic literature review of the pigments produced by this actinomycetes genus was carried out. The main bioactivities reported, such as antimicrobial, antioxidant, and cytotoxic, were identified and are relevant to determine possible future applications. Subsequently, the identified and unidentified pigments were classified with their respective yields to make an overall economic analysis of the factors that may affect pigment extraction and purification. In addition, the study strains' conditions for optimizing pigment production were summarized. This systematic review aimed to identify, summarize and evaluate the evidence regarding the potential of *Streptomyces* strains as a biological source of bioactive pigments.

2.3 MATERIALS AND METHODS

2.3.1 Databases and Search Strategy

The search was performed using the following databases: Scopus, Web of Science, and PubMed to review the literature as complete as possible. The terms (including synonyms and related words) and Boolean operators used for all searching were defined as follows: *streptomyces* AND (pigment OR colorant OR stain OR dye OR coloring OR tint).

2.3.2 Selection Procedure

The selection of the articles was based on the following inclusion criteria: (a) original research articles, (b) studies on extracts, compounds, fractions, or pure culture derived from *Streptomyces* strains, and (c) studies related to pigment production (evaluation of bioactivities, purification or/and elucidation, optimization of the production).

The following were considered exclusion criteria: (a) articles were written in a language other than English, (b) articles are not available publicly, and (c) the main objective of the article is not related to the production of the pigment.

The article selection process was subdivided into two stages: In the first stage, four researchers separately assessed each title and abstract in a blind process. At this time,

each article was marked as potentially eligible to be included in the review when at least two studies indicated that it met the inclusion/exclusion criteria. When a piece was suggested as suitable by only one researcher, a discussion within the research team was carried out to solve the disagreement. In the second stage, potentially eligible articles were examined at the full-text level. Thus, those articles that comply with the inclusion/exclusion criteria were finally selected for data extraction.

2.3.3 Data Collection and Tabulation

A pilot data acquisition form was prepared to guarantee careful data collection and avoid the risk of bias. The paper was evaluated and improved through an exercise including ten articles selected by chance. In this manner, having defined the definitive version of the state was used for data acquisition of the complete set of selected articles. The data were tabulated using the paper between two researchers and verified jointly.

2.3.4 Structure-Based Clustering

The pigmented compounds retrieved from this systematic review were converted to SMILES notation (simplified molecular-input line-entry system) using Marvin JS (ChemAxon, Budapest, Hungary) to build a custom-made library. The above, for analyzing the structures of bioactive pigments and classification within the diverse types of biological pigments.

2.3.5 Data Analysis

A first analysis of the methodologies and results of the articles included the main bioactivities evaluated. Additionally, a meta-analysis was conducted with the metabolites retrieved from the included pieces to relate the structural profile and some functional features for a potential application in cosmetic, food, pharmaceutical, or other industries. Accordingly, some standard physicochemical parameters were selected, and structure-activity relationship analysis (SAR) was performed using Osiris DataWarrior v5.2.1.

2.4 Results and Discussion

2.4.1 General findings

The literature search identified 3522 articles, of which 1172 were duplicates, giving 2350 articles. These articles were screened by reading titles and abstracts by inclusion or exclusion criteria; from this stage, 101 papers were selected for full-text evaluation. Finally, 42 articles were selected for full-text assessment and used for data extraction. Figure 1, resume the identification of studies.

Figure 2 shows that 92.9% of the journals of the selected articles are indexed in the SCImago Journal Rank (Figure 2B), and most of the journals nonindexed were discontinued. Moreover, 66.7% of the indexed journals are included in the Q1(38.5%) and

Q2 (28.2%) classification, considering the best quartile of each journal (Figure 2C). Considering the above, the high quality of the articles in this line of research is evident.

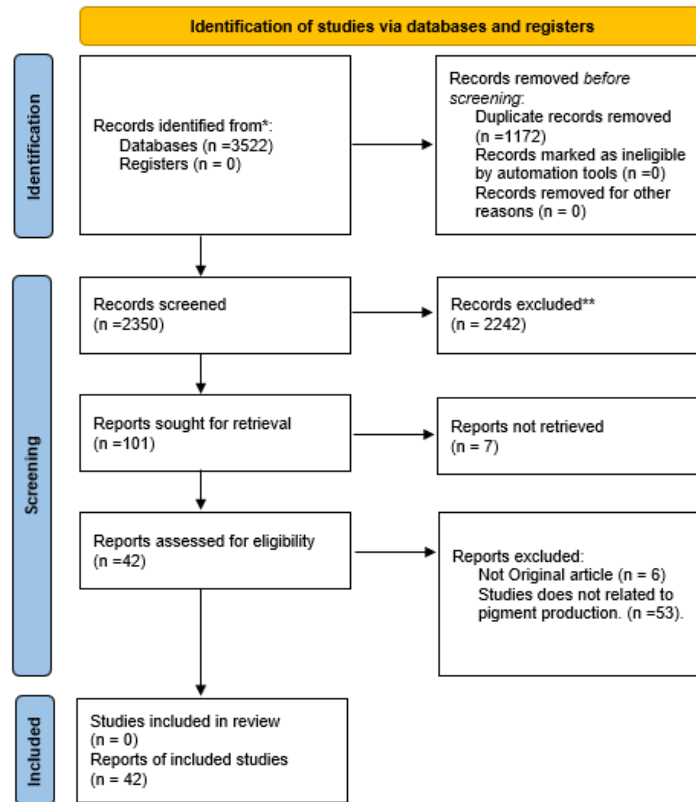


Figure 1 PRISMA flow diagram. Flowchart of systematic literature search according to PRISMA guidelines. It is modified from Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for the reporting system.

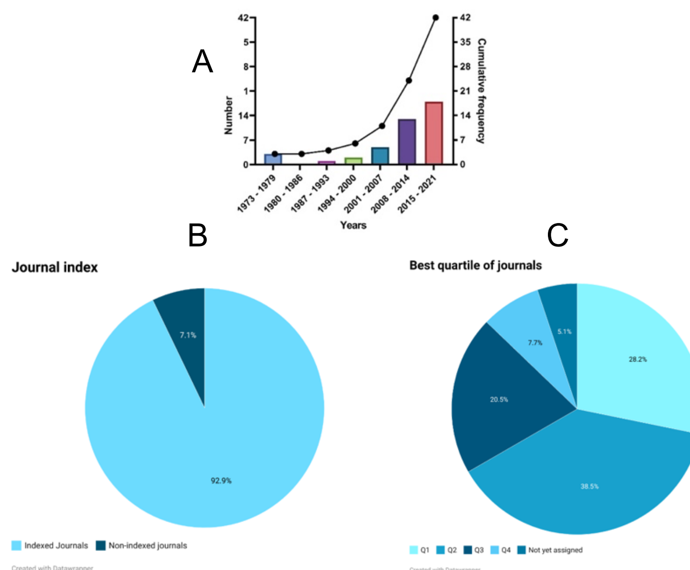


Figure 2 General findings around the journals of the selected articles. A) Publication distribution over time. The first y-axis shows the number of articles, and the second y-axis shows the cumulative frequency distribution. B) Percentage of articles that are indexed or non-indexed. C) Percentage of articles by best quartile of journals.

The map of the countries of the correspondence authors of the articles (Figure 3) shows that the contribution of reports from Latin America, Europe, and Africa is extremely limited or almost null. As for the leading countries, India stands out as the country with the most outstanding academic contribution, intricately linked to the *Streptomyces* collection areas, as seen in Figure 4. This way, Asia is the main continent where the scientific production and collection sites around *Streptomyces*-derived pigments are concentrated. Also, 23.3% of the articles do not specify the isolation source.

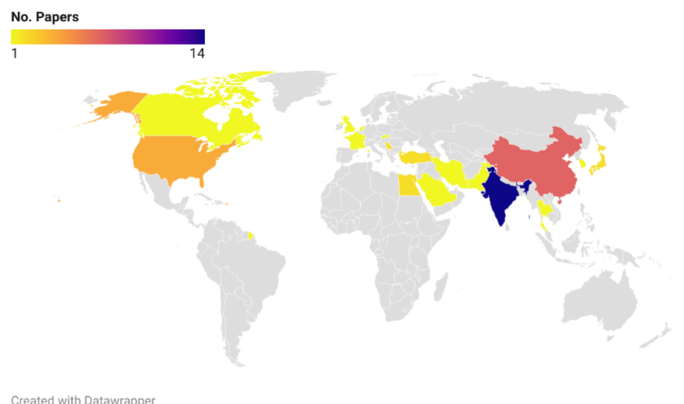


Figure 3 World map showing where the articles included in this review were produced and the corresponding author affiliation.

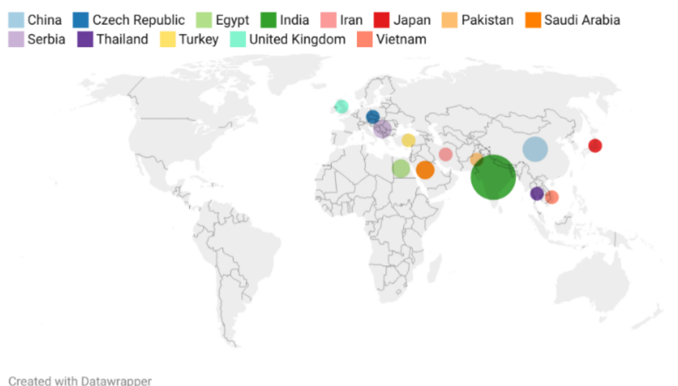


Figure 4 World map showing the countries where the Streptomyces strains were isolated.

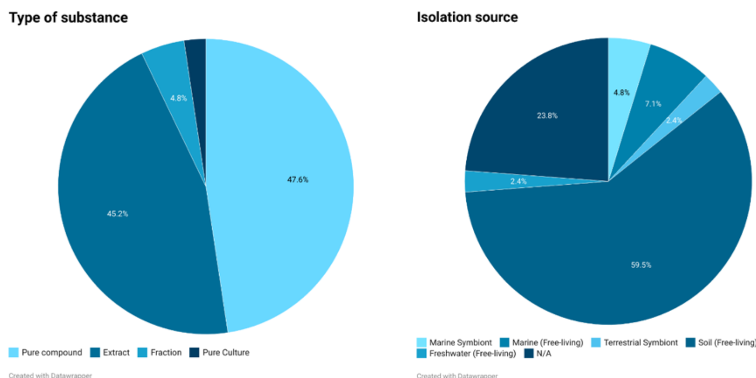


Figure 5 Characteristics of the information registered in the selected articles. A) Percentage of articles evaluated either crude extracts, pure compounds, fraction, or pure culture. B) Percentage of articles by the environment where the isolation occurred.

Figure 5 shows the percentage of the type of substance under study. Of the articles evaluated, 47.6% reported working with pure compounds, 45.2% worked with the extract, and the rest, corresponding to 7.2%, evaluated the extract and the pure culture together. On the other hand, according to the source type, it was found that the most used solvent for the extraction and purification of the extract was ethyl acetate; other solvents used were methanol, chloroform, and ethanol. As for the source of isolation, 59.5% belonged to soil (free-living), 7.1% to marine (free-living), and 4.8% to marine symbionts, in smaller quantities terrestrial and freshwater symbionts (free-living) with 2.4%. In addition, 23.8% of the articles do not report the source of isolation.

The most-reported areas of *Streptomyces* collection in India have sediment characteristics, highlighting leaf litter and marine soils; among them is the Vellar, Tamilnadu, as reported by four articles. Another of the protagonist areas has been the Thar, Rajasthan, which has reported yellow pigments. The same happens in China, the second country with more articles based on free-living sediments, especially from the provinces of Henan and Nanjing.

When analyzing the number of pigment-producing strains from different isolation sources and the evaluated bioactivities, it is observed that the highest number of reported strains are from the soil (Free-living), with 26 reported strains and the most evaluated bioactivity was antimicrobial activity. In addition, seven pigment-producing strains from soil were evaluated for more than one bioactivity (Table 1).

On the other hand, the isolated source with the fewest reported pigment-producing strains is Terrestrial Symbiont, and the only one said it was not evaluated for any bioactivity. In addition, the bioactivity with the most reported strains is antimicrobial activity, and 19 pigment-producing strains were not evaluated for any bioactivity (Table 1).

Table 1 Number of strains and compounds by bioactivity and type of source. [a] Information not available.

Source	Bioactivity	No. Strains	No. Compounds	Ref.
Freshwater (Free-living)	Antimicrobial	3	3	23
Marine (Free – living)	Antimicrobial	4	1	24,25
	Antioxidant	1	1	26
Marine Symbiont	Cytotoxic	1	0	27
	N/A	1	0	28
Terrestrial Symbiont	N/A	1	3	29
Soil (Free – living)	Antimicrobial	9	1	30-38
	Antioxidant	1	1	39
	Cytotoxic	3	1	40-42
	Multiple [c]	8	6	43-50
	N/A	5	1	51-54
N/A[a]	Antimicrobial	3	4	55-57
	Antioxidant	1	1	58
	None	12	4	59-64

2.5 Bioactivity results

2.5.1 Antimicrobial activity

The main methodology to evaluate the antimicrobial activity was the disk diffusion method. Different microorganisms were used to evaluate the antimicrobial activity: gram-negative bacteria such as *E. coli* [50]–[53], *Salmonella* sp. [41], [46]. *K. peumoniae* [15], [54]–[56], and *P. aeruginosa* [56], [57] (Table S1); gram-positive bacteria such as VRSA (vancomycin-resistant *Staphylococcus Aureus*) [47], *Enterococcus sp*. [46], [58], *Nocardia asteroides* [59](Table S2); fungi such as *Aspergillus niger* [45] and *Fusarium oxysporum* [57], and yeasts such as *Saccharomyces cerevisiae* [60] (Table S3). Many of the pigment-producing *Streptomyces* strains have antimicrobial activity reporting zone of inhibition measurement (Table 2), and, in other cases, minimum inhibitory concentrations (MIC) were determined (Table 3).

Table 2 Pigment-producing Streptomyces strains with reported antimicrobial activity.

Strains	Positive antimicrobial tests	Ref.
Streptomyces* sp. *F1 ***Streptomyces* sp. *F2*** ***Streptomyces* sp. *F3***	*E. coli, Lactobacillus vulgaris, Proteus mirabilus, Vibrio cholera, S.aereus, S. typhi, S. paratyphi, K. oxytoca*	[41]
Streptomyces coeruleorubidus NBRC 12844	*S. aureus (ATCC 1112), B.cereus (ATCC1015), P.aeruginosa (ATCC 1074), C. freundii (ATCC 8090), K.pneumoniae (ATCC 1053), S. marcescens (ATCC 14756)*	[45]
Streptomyces parvulus C5-5Y	*S. aureus, S. epidermidis, Enterococcus* sp. *,E. coli, P.aeruginosa, K. pneumoniae, S. typhi, Proteus* sp. *,Shigella* sp., *Bacillus* sp.	[46]
Streptomyces D10	*MRSA, VRSA, E. coli and Klebsiella sp.*	[47]
Streptomyces* sp. *D25	*M. tuberculosis H37Rv and MRSA*	[51]
Streptomyces* sp. *S45	*S. aureus ATCC 29213 and B. cereus*	[52]
Streptomyces SAG-85	*E. coli, S.aureus, MRSA, K. pneumoniae, P.aeruginosa, E.faecalis, S. marcescens and Salmonella* sp.	[61]
Streptomyces* sp. *SFA5	*M. tuberculosis H37Rv*	[62]
Streptomyces sp. 24	*P.aeruginosa ATCC 9027, E.coli ATCC 8379, S. aureus ATCC6538, Mycobacterium smegmatis ATCC 10231*	[57]
Streptomyces* sp. *BSE6.1	*S. aureus MTCC1430*	[63]
Streptomyces peucetius ATCC 29050	*B. subtilis.*	[64]

Table 3 Pigment-producing Streptomyces strains with antimicrobial activity reported minimal inhibitory concentration (MIC).

Streptomyces Strain.	Positive antimicrobial tests	MIC(µg/ml)	Ref.
***Streptomyces* sp. (MVCS13)**	*Bacillus* sp. *FPO1*	23	[40]
	Aeromonas sp. *FPO2*	27	
	Citrobacter sp. *FPO3*	21	
	Edwardsiella sp. *FPO4*	20	
	Vibrio sp. *FPO5*	18	
	Aeromonas sp. *FPO6*	22	
S. spectabilis strain L20190601	*Staphylococcus aureus*	0.25	[51]
	Bacillus subtilis	0.5	
	Escherichia coli	4	
	Streptococcus pyogenes	0.5	
	Pseudomonas aeruginosa	0.5	
	Bacillus typhi	1	
	Candida albicans	2	
	Trichophyton rubrum	64	
Streptomyces* sp. *JS520	*Micrococcus luteus ATCC 379*	50	[58]
	Bacillus subtilis ATCC 6633	50	
	Candida albicans ATCC 10231	100	
	Candida albicans ATCC 10259	200	
Streptomyces* sp. *strain JAR6	*Salmonella* sp.	150	[54]
	Bacillus subtilis	50	
	Proteus mirabilis	80	
	Shigella sp.	100	
	Escherichia coli	170	
	Enterococcus sp.	120	
	Klebsiella pneumoniae	180	
Streptomyces aurantiacus AAA5	*Bacillus subtilis*	25	[56]
	Staphylococcus aureus	13	
	S. epidermis	8	
	Enterococcus faecalis	5	
	Klebsiella pneumoniae	16	
	Shigella sp.	45	
	Proteus vulgaris	70	
	Escherichia coli	42	
	Pseudomonas aeruginosa	34	
	Salmonella typhii	15	

Table 3 shows that *Streptomyces* pigments or colored extracts from different sources have high antimicrobial activity against the genus Bacillus, as it is the most frequent microorganism in the antibacterial bioactivity assay. In addition, its potential is very versatile at MIC concentrations since 100% mortality of the pathogen can be obtained at

0.5, 1, 25, and 50 µg/mL. On the other hand, relevant antimicrobial bioactivity is also observed in E. coli, the second most frequent bacterium, showing sensitivity to *streptomycetes*, reporting MICs of 4, 42, and 170 µg/mL.

2.5.2 Antioxidant activity

The second most evaluated bioactivity was antioxidant activity, and some widely used methods such as DPPH, ABTS, reducing power assay, 9Hydroxyl, and superoxide radical scavenging activity; other less common methods such as ferric thiocyanate method, lipid peroxidation, and protein oxidation inhibition assay. On the other hand, different types of results have been reported for antioxidant activity: IC_{50} values and equivalence to vitamin C (Table 4), the percentage at a specific concentration (Table 5), and some descriptive results (Table 6).

Table 4 Results of pigment-producing Streptomyces strain with reported antioxidant activity in $IC50$ and vitamin C equivalence.

Streptomyces strain	Antioxidant method evaluated	$IC50$ (µg/mL)	Equivalence to vitamin C (µg)	Ref.
Streptomyces sp. A1013Y	DPPH	41.04	0.493	[65]
	ABTS	13.75	1.12	
Streptomyces glaucescens KCTC988	ABTS	25080	N/A	[66]
	ABTS (In presence of copper ions)	7890	N/A	

Table 5 Results of pigment-producing Streptomyces strains with reported antioxidant activity, expressed in % radical uptake, with their respective concentrations evaluated.

Streptomyces strain	Antioxidant method evaluated	Concentration evaluated (µg/mL)	Percentage of activity	Ref
Streptomyces sp.	Hydroxyl radical scavenging activity	500	70.24%.	[67]
Streptomyces glaucescens NEAE-H	ABTS	100	57.20%	[15]
Streptomyces parvus BSB49	DPPH	250	87.73%	[68]
	ABTS	250	75.20%	
Streptomyces sp. WMA-LM31	DPPH	10	60.50%	[69]
	Lipid peroxidation inhibition assay	10	25.42%	
	In-vitro protein oxidation inhibition assay	10	54.82%	
Streptomyces sp. 24	DPPH	5	65.30%	[57]
	Hydroxyl radical scavenging activity	50	96.20%	
	Superoxide scavenging activity	10	42.80%	
		50	59.90%	

Table 5 reflects the most applied methods to evaluate the antioxidant activity of *Streptomyces* pigments, which correspond to DPPH and ABTS, evaluating concentrations of 5, 10, 250, and 100 µg/mL and giving percentages of free radical uptake very similar or close in order of magnitude. Such is the case of *Streptomyces parvus* BSB49, which at a concentration of 250 µg/mL, using DPPH, had bioactivity of 87.3%, and with ABTS, it was 75.20%.

Table 6 . Descriptive results of the pigment-producing Streptomyces strain with reported antioxidant activity were reported.

Streptomyces strain	Antioxidant method evaluated	Results of antioxidant activity	Ref.
Streptomyces sp.	Superoxide radical scavenging activity	Moderate scavenger of superoxide radical in vitro and exhibited a strong dose-effect relationship.	[67]
	Reducing power assay	Antioxidant activity of melanin might be due to redox reactions	
Streptomyces sp. JS520	Ferric thiocyanate method	Undecylprodigiosin did not perform as well as commercially available antioxidant α-tocopherol; however, it was effective in delaying lipid peroxidation.	[67]
	Hydrogen peroxide disc diffusion assay	Undecylprodigiosin is acting as scavenger of H2O2 that is released through the process of peroxidation.	
Streptomyces sp. strain JAR6	DPPH	Strain JAR6 was able to reduce compounds to pale yellow hydrazine as DPPH radical.	[54]
Streptomyces coelicolor MSIS1 (c)	Reducing Power Assay	The pigment has positive results for all the concentrations 10mg/ml, 50 mg/ml and 100mg/ml.	[55]

2.5.3 Cytotoxicity activity

For the evaluation of cytotoxic activity, cancer cell lines such as fibrosarcoma (HT1080), larynx (Hep2), cervix (HeLa), breast (MCF7), liver (HepG2), skin (HFB4), human nasopharyngeal carcinoma (KB cells) and non-cancer cell lines, such as human lymphocytes, peripheral blood mononuclear cells (PBMC), human lung fibroblasts (WI-38), human amniocytes (WISH) were used. (Table 7) In most cases, cytotoxic activity is reported using the half-maximal inhibitory concentration (IC_{50}) in concentration units (µg/mL and µM), which is the amount of a specific drug needed to inhibit a biological process by half [70] (Table 8).

Table 7 IC 50 (µg/mL) of Streptomyces-derived pigments and their conditions (Cell lines, cell density, among others). Ac=any cytoxicity, Lc*=Low cytotoxicity*

***Streptomyces* Strain**	**Cell density (cells/well)**	**Concentration (µg /ml)**	**Pigment**	**Time of treatment (h)**	**Cell line**	**IC 50 (µg/mL)**	**Ref.**
Streptomyces sp. PM4	$2x10^4$	10, 20, 30, 40, 50	Red pigment	24	HT1080	18.5	[42]
					Hep2	15.3	
					HeLa	9.6	
					MCF7	8.5	
Streptomyces griseoaurantiacus JUACT 01	N/A	2.5, 5, 10, 20	Yellow Pigment	24	HeLa	5.31	[71]
				48		2	
				72		1.8	
				24	HepG2	26.33	
				48		1.75	
				72		1.41	
				24, 48, 72	Human lymphocytes	Ac*	
Streptomyces A 16-1	$5x10^4$	0 - 8	Red pigment (Fr 5, Fr6, Fr7)	48	KB cells	0.04 (Fr 5)	[72]
						0.20 (Fr 6)	
						0.55 (Fr 7)	
					PBMCs	Lc*	
Streptomyces sp. strain JAR6	$1x10^4$	18.75, 37.5, 75, 150, 300	Red pigment (Undecylprodigiosin)	48	HeLa	145	[54]
Streptomyces glaucescens NEAE-H	$1x10^4$	1.56, 3.125, 6.25, 12.5, 25, 50, 100	Melanin	24	HFB4	16.34	[15]
					WI-38	37.05	
					WISH	48.07	
Streptomyces sp. WMA-LM31	$1x10^4$	5, 10, 15, 20	Prodigiosin	24	HepG2	12.66	[69]
					HeLa	14.83	

In Table 7, the most used cell density is $1x10^4$ cells per well, and the most common concentrations are 5-20 µg/mL. In addition, the red pigments have the highest cytotoxic activity, especially against HeLa cells. This is a very attractive result, considering the importance of the HeLa cell line in the scientific world, given its attributes in the development of many drugs, and vaccines to combat viruses and terminal diseases caused by cancer [73].

A result that also adds to the cytotoxic activity against HeLa cells is reported in *Streptomyces parvus BSB49*, whose cell density per well is $3x10^4$, evaluating concentrations of 5, 10, and 20 µM, the pigment identified corresponds to Eumelanin, with an IC_{50} of 10 µM [68].

In other cases, cytotoxic activity is reported using different measures such as growth inhibitory activity (GI_{50}), which is the concentration of the tested compound required to cause a 50% decrease in net cell growth [74]; and lethal concentration 50 (LD_{50}) which is the concentration of a given agent where 50% cell lethality is obtained (Table 8) [75].

Table 8 GI50 and LC50 of Streptomyces-derived pigments and their conditions (Cell lines, cell density, among others)

***Streptomyces* Strain**	**Cell line**	**Cell density (cells/well)**	**Concentration (µg /ml)**	**Time of treatment**	**Pigment evaluated**	**GI50 (µg/mL)**	**LD50 (µg/mL)**	**Ref.**
Streptomyces aurantiacus AAA5	HepG2	N/A	5, 0.5,0.05,0.005, 0.0005	N/A	Resistomycin (Yellow)	0.005	0.01	[56]
	HeLa	N/A		N/A		0.006	0.013	

Another way to evaluate cytotoxic activity is in vivo assays. The reported measure is the median lethal dose (LD_{50}), as reported in *Streptomyces coelicolor 100*, whose assay consisted of an acute toxicity treatment in mice. The test was divided into two phases, evaluating different concentrations in mg/kg and obtaining results of an LD_{50} > 15,000 mg/kg.

2.5.4 Pigment purification

In 90.5% of the selected articles, partial or complete purification of pigments is described. Thus, the purification of *Streptomyces* pigments is not standardized; however, among the most applied methods in the literature review, the following techniques are found: centrifugation, different types of chromatography, pressure conditions, use of reagents, lyophilization, and thermal processes. The initial process, in most cases, includes centrifugation to remove the biomass [40], [72], and the rest of the process is used to remove the supernatant where the pigment is solubilized [69], [76] or to keep the pellet where the insoluble pigment is precipitated [57], [66].

Types of chromatography used for pigment purification include flash chromatography [58], column chromatography on alumina [59], thin layer chromatography (TLC) [44], [47], [59], ion exchange chromatography [40]high-performance liquid chromatography (HPLC) [43], [55], high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [51], semi-preparative HPLC [51], liquid chromatography-mass spectrometry (LC-MS) [56]. Thus, some of the columns used include the SephadexG-50 [40], [67], the silica gel column [42], [72], the Kromasil ODS C-18 column [76], the Sephadex LH-20 [69], [77], [78], the Zorbax ODS C-18 [54], silica-coated glass plates [79], silica gel-coated aluminum foils [63], and the DEAE-Sepharose column [64].

Different types of reagents, such as acids or bases, are used to modify the pH and precipitate the target pigment [15], [40] ; or solvents in different proportions (organic, polar, nonpolar) to extract the pigment from the fermentation broth [52] or to establish a solvent system [71] Different procedures that modify the pressure or temperature are also

used to concentrate the pigment, such as vacuum freeze drying or freeze-drying [15], [16], [41], flash evaporation [41], [80] or at reduced pressure [42], [63], and rotary evaporator [62], [63].

Less commonly used methods for pigment purification include the ultrasonic cell disruption system for lysis [16] or sonication [69], membrane filtration [60] and ultrafiltration membrane [64], the use of a Centricon-30 concentrator [64], and a Soxhlet extractor [81]. Another variable modified to increase purification is the number of times a specific procedure is repeated. For example, performing centrifugation two or three times [41], [78], [82], performing re-extraction many times [42], [65] and repeated crystallizations [81]. Even in special cases, the purity of the separated compounds has been detected using a UV illuminator [46].

On the other hand, liquid-liquid extraction [62] and solid-liquid extraction are used. In the latter case, the crude pigment secreted to the agar was extracted using different organic solvents [48], or the mature sporulated aerial mycelium was extracted with ethyl acetate twice. Thus, some of the selected articles describing pigment purification reported the yield based on the dry weight of pigment from the initial volume of the fermentation broth. (Table 9).

Table 9 Yield reported each type of pigment and the Streptomyces strain that produces it.

Streptomyces **Strain**	**Type/Color of pigment**	**Yield reported (mg/L)**	**Ref.**
Streptomyces sp. 13-4	Streptorubrin B (Prodiginine pigment)	2	[59]
Strptomyces sp. CWW6	Streptorubrin A (Prodiginine pigment)	20	
Streptomyces sp. (MVCS13)	Melanin	238.57	[40]
Streptomyces sp.(F1,F2,F3)	Melanin	21130	[41]
Streptomyces sp.	Melanin	1460	[16]
Streptomyces sp. PM4	Red pigment	1874	[42]
Streptomyces acidiscabies	Bright yellow compound (1)	63	[44]
	Orange compund (2)	28	
	Compund (3)	2	
Streptomyces sp D25	Yellow pigment	175 to 1225	[48]
Streptomyces sp. A1013Y	Blue pigment	2	[65]
Streptomyces griseoaurantiacus JUACT 01	Yellow pigment	4300	[71]
S. coelicolor 100	Blue pigment	3000	[76]
Streptomycessp. JS520	Red pigment	138.94	[58]
Streptomyces glaucescens NEAE-H	Melanin	350	[15]
Streptomyces parvus BSB49	Eumelanin	160 to 240	[68]
Streptomyces sp. WMA-LM31	Prodigiosin (Red)	30	[69]
Streptomyces coelicolor MSIS1 (c)	Red or blue depending on conditions	5030 (shake flasks), 9000 (bioreactor)	[55]
Streptomyces aurantiacus AAA5	Resistomycin (Yellow compound)	52.5	[56]
Streptomycessp. 24	Melanin	59.3 to 138	[57]

Streptomyces coelescens ATCC 19830 (NP2)	Deep blue	3000 to 4000	[83]
Streptomyces anthocyanicus ATCC19821 (NP4)	Deep red	3000 to 4000	
S. griseus IFO13350 w	Grixazone A (Yellow)	4.7	[77]
	Grixazone B (Yellow)	2	
Streptomyces glaucescens KCTC988	Melanin	125.25 +/- 6.01	[66]

As can be seen in Table 9, the melanin family reports the highest pigment production yield, reaching 21130 µg/mL, by *Streptomyces* sp. (F1, F2, F3). In second place is the red pigment from *Streptomyces coelicolor MSIS1*, going 9000 µg/mL in bioreactor fermentation. As for yellow pigments, yields ranging from 59 to 1225 µg/mL can be obtained.

2.5.5 Optimization of the pigment's production

One of the main concerns in pigment production is the yield; for this reason, the optimization of culture media and fermentation conditions becomes important. There are different ways to improve the amount of pigment: changing the type of medium used, including ISP (International *Streptomyces* Project) medium, among others; changing the concentration of the components of a base culture medium; changing the main nutrient sources such as carbon and nitrogen sources; even using experimental designs such as Plackett-Burman and central composite design with multiple factors and levels [15], [42], [57].

The main variables most optimized are the carbon and nitrogen sources and their concentration: Carbon sources include glycerol [40], starch, dextrose, maltose, maltose, fructose, glucose [41], [42], xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol, sucrose [43], lactose [45], melibiose, and sorbose [50]. Nitrogen sources include L-tyrosine or tyrosine, asparagine [40], [42], yeast extract, soybean meal, peptone [41], [42] , ammonium sulfate, malt extract, phenyl alanine, histidine [42], glutamine [48], protease-peptone potassium nitrate, ferric ammonium citrate [15], sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl), beef extract, casein, casein peptone, meat peptone [54], soybean peptone [57], KNO_3 [84], ammonium nitrate [85] and urea [80]. Considering the economics and a large amount of agro-industrial waste, the use of new nutrient sources such as sugarcane residues, rice bran, wheat bran, coconut cake, and rice flour [41]. Other components of culture media that have been used to optimize pigment production include salts such as $MgSO_4$, $NaCl$, $FeSO_4$, KH_2PO_4 [24], Na_2HPO_4 [42], $CaCO_3$, KCl [48], sodium thiosulfate [15], KH_2PO_{45}, $CaCl_2$, $NiCl_2$ [50], $FeCl_2$, $MgCl_2$, $ZnCl_2$, $MnCl_2$, $CaCl_2$, $COCl_2$ [80] and amino acids such as glycine, cysteine, alanine, tryptophan, valine [43], tryptone [45], leucine, proline, glutamine [85], aspartate and glutamate [80]. Other variables taken into account to optimize pigment production are pH (5-9.5), temperature (10-60°C) [40], [41], [45] , salinity (0-20ppt) [41], NaCl concentration (1-10%) [43], [48], [86], incubation period (3-15 days) [54], agitation speed (50-200 rpm) [58], medium volume (ml/250ml flask) [15], inoculation size [57], sources and phosphate concentration [85].

Table 10 Optimization conditions of pigments produced by each Streptomyces strain.

Strain	Optimized variable	Optimization result	Referencia
***Streptomyces* sp. (MVCS13)**	Temperature	50°C	[40]
	pH	7.4	
	L-Tyrosine	0.75 g/L	
	Aspar-agine	1.5 g/L	
	$MgSO_4$	0.25 g/L	
	$NaCl$	0.75 g/L	
	$FeSO_4$	0.015 g/L	
	Trace Salt solution	1.5 ml/L	
***Streptomyces* sp.(F1,F2,F3)**	Carbon Source	starch 1%w/v	[41]
	Nitrogen source	soyabean 0.2%w/v	
	Salinity	15 ppt	
	Temperature	35°C	
	pH	7	
	incubation time	168 h	
	Cheaper source	Sugarcane waste	
***Streptomyces* sp. PM4**	Carbon Source	maltose (4.06 g/L)	[42]
	Nitrogen source	peptone (7.34 g/L)	
		yeast extract (4.34 g/L)	
		tyrosine (2.89 g/L)	
***Streptomyces* strain (AQBWWS1)**	Carbon Source	Glucose	[42]
		Xylose	
	Aminoacids	Cystine	
		Tryptophan	
	NaCl Concentration	2.50%	
***Streptomyces* sp. D25**	Carbon Source	Glucose	[48]
		fructose	
	Nitrogen source	Malt Extract	
	pH	7,9,11	
	Temperature	30°C,40°C	
	NaCl Concentration	1-5%	
***Streptomyces* sp. S45**	Carbon Source	Glucose	[52]
		Rhamnose	
	Nitrogen source	soybean meal	
	Minerals	CaCl2	
	pH	7	
	Temperature	30°C	
***Streptomyces glaucescens* NEAE-H**	Incubation period	6 days	[15]
	Nitrogen source	protease-peptone (5g/L)	
		ferric ammonium citrate (0.5g/L)	
***Streptomyces* sp. 24**	$NiCl_2$	3.05 mM	[57]
	$FeSO_4$	1.33 g/L	

	Soy peptone	20.31 g/L	
	pH	7	
	Temperature	30°C	
	inoculation size	3% (v/v)	
	Incubation period	5 days.	
Streptomyces indigocolor	Carbon Source	Glucose	[87]
	Nitrogen source	KNO 3	
Streptomyces canaries	NaCl Concentration	>10%	[86]

2.6 DISCUSSION

Streptomyces is an exciting and relevant source for the scientific community and the biotechnology industry [54]. Pigments, one of the most attractive secondary metabolites due to the multiple applications and bioactive potential they can bring to many industries [88], have gained strength in recent years, as seen in Figure 2. This systematic literature review highlights the extraordinary opportunities that developing new pigments, their documentation, and subsequent production would bring to the industry. Current pigments are the focus of concern due to the adverse effects reported for human health and their impact on the environment due to their synthetic and unsustainable origin [89].

Although the study of *Streptomyces*-derived pigments goes back many years, starting in 1973, the most significant number of articles (85.7%) was published between 2001 and 2021. This evidences the relevance that the production and evaluation of *Streptomyces-derived* pigments has taken, given the growth in the number of articles published in recent years (Figure 2A). This phenomenon can be explained by the inclusion of a greater number of countries other than the United States, Canada, the United Kingdom, and the Netherlands in this area of research. However, Figure 3 shows the lack of academic contribution from Latin America and Africa. This could be explained by the fact that there is very little intellectual development in this area of *Streptomyces*-derived pigments. Additionally, it is known that there is no consistent line of research on a specific research topic in Latin America, according to the study conducted by Corrado Nai [90]. This could also apply to Africa or be attributed to economic resources since many techniques or experimental designs to evaluate such complex compounds as pigments require specialized equipment with a high research cost [91], [92]. This low contribution of *Streptomyces* pigments in Africa and Latin America can be considered an area of interest to diversify the *Streptomyces* genus further. Additionally, explore the discovery of new color ranges. The benefits of obtaining *Streptomyces* pigments from these soils promote the economic development of these regions and increase the scientific knowledge of this microbial genus [93].

As for the area of most excellent collection and scientific production, there is a strong influence on the Asian continent, as shown in Figure 4. It is worth mentioning that Asia is a source of microbial biodiversity since it is the longest continent in the world and contains most of the morphological extremes of the earth's surface [94]. *Streptomyces* are known for quickly adapting to nutrient-starved, unfriendly, hostile, or exotic environments [95].

Another aspect to highlight in this literature review, according to the general results, is the type of substance reported in the classified articles. Figure 5 shows that most of the research led to extract (45.2%) and pure compound (47.6%). Table 9 summarizes the type of pigment reported by the articles.

The most-reported pigments from different *Streptomyces* strains are prodigiosin, melanin, and prodiginin. This allows inferring that most of the research on *Streptomyces* pigments is still based on molecules that already have a long research history. On the other hand, the type of collection habitat could attribute to the frequency of color found. However, a more detailed analysis of each strain reporting the kind of color shows, for example, *Streptomyces* sp 13-4 was collected from a marine habitat. In contrast, *Streptomyces CWW6* was collected from sediments, and both yielded prodigiosin pigment. The same is true for melanin, which is the most common pigment. *Streptomyces* sp. strain (MVCS13), *Streptomyces glaucescens* strain NEAE-H, and *Streptomyce*s sp. (F1, F2, F3) were collected from totally different environments and countries. Therefore, it can be speculated that the collection source is independent of the reported color frequency and that different color ranges can be obtained from one source. However, yes, it could be presumed that color determination is influenced by habitat composition and the genetic complexity of the microorganism [96].

This complex genetic configuration and the composition of the collection source is strongly linked to the type of bioactivity expressed by *Streptomyces* [96]. In Table 1, it is observed that 26 *Streptomyces* strains were collected from sediments, and their main activity was antimicrobial activity. This can be explained from the soil adaptation perspective of *Streptomyces* [96]. Generally, this genus of *actinomycetes* grows as a vegetative mycelium, which matures into multiple hyphae, which spread and branch in search of nutrients. In this process, a large production output of enzymes which decompose numerous organic compounds. Therefore, the production of these metabolites serves as tools to kill other types of microorganisms living in the soil or modulate the transcription and gene transfer of the same, thus controlling their bioactive response [96].

This review is of great interest in line with what is reported in Table 1. It can be observed that the most reported bioactivities correspond to antimicrobial, antioxidant, and cytotoxic activity. This implies that these are the minimum activities required to determine the safety of pigments from microbial material, to give them the green light in medical, cosmetic, and food applications, among other areas, which may endanger human health [97], [98].

Further elaboration of the reported bioactivities. Table 2 summarizes the most common microorganisms showing sensitivity to *Streptomyces* pigments, either in extract or pure compound format, with their respective MICs. Ranges from 0.5 to 200 ug/mL are observed. This reaffirms the versatility of *Streptomyces* that, depending on the conditions, genetics, and composition of the medium in which it has been cultivated, pigmented secondary metabolites with high antimicrobial potential can be obtained. On the other

hand, it can be highlighted that these colored antibiotics possibly have the presence of antimicrobial peptides [99]. These therapeutic agents may explain the relationship observed in many *Streptomyces* strains, which report antimicrobial and cytotoxic activity [99]. Studies suggest a correlation between antimicrobial and cytotoxic activity due to the complex electrostatic interaction that occurs with the bacterial or cell membrane that allows inducing the inhibition of the pathogenic microorganism or the cell death of neoplastic cells [99].

As for the antioxidant activity, Tables 4 and 5 can be reviewed. Many of the pigments reported had a better antioxidant effect than the globally recognized molecules, among which Vitamin C, Tocopherol, and Trolox, among others, stand out [100]. However, only two strains reported IC_{50} in mg/mL equating to µg in Vitamin C. It is essential to highlight that antioxidant compounds are of great interest and usefulness due to their benefits to human health [101].

Therefore, it would be beneficial to map more IC_{50} of the antioxidant activity of the pigments produced by *Streptomyces* to analyze more precisely the influence of the technique applied and the components present in these colored extracts in Table 5; there is no standard methodology when evaluating antioxidant activity. This is almost impossible because of the multiple mechanisms of action a molecule must capture free radicals, and there is a risk of losing important information [101]. However, it is possible to extend the suggestion through the analysis carried out with this review to propose a standard methodology to evaluate the antioxidant activity in microbial pigments. Where at least two or three techniques with different principles can be included. As in the case of the ABTS, whose purpose is to measure the capacity to eliminate radicals, the SOD determines the inhibition of the superoxide anion radical, and the chelating agent assay measures the ability to chelate metal ions [101]. This would allow a broader view of the actual antioxidant capacity of a pigment produced by *Streptomyces*.

Finally, the last biological activity reported is cytotoxic activity. Nowadays, the search for natural products that prevent cancer development is becoming more and more relevant [101]. Color molecules that have a cytotoxic effect against cancer cells make them very attractive metabolites for the scientific community [101].

Tables 7 and 8 summarize the *Streptomyces* pigments that report cytotoxic effects against different types of cancer cell lines. Something very particular that can be observed in the reported pigments is that most of them are red, and the most common cell line in which they were evaluated corresponded to HeLa. Red stains, such as prodigiosin stand out for being part of a family of oligopyrrolyl-type antibiotics [102]. Several studies have reported the antitumor, anticancer, and immunosuppressive activity of these secondary metabolites produced by *Streptomyces* and other types of microorganisms [102]. Its bioactivity may be related to the type of chemical configuration that this type of complex molecule has since it may have the presence of linear pyrroles, macrocyclic compounds, and cyclic compounds of a single pyrrole [102].

Another aspect to highlight is the cell density at which the pigments were evaluated and reported their respective IC_{50}, LD_{50}, and GI_{50}. It could be observed that *Streptomyces* A 16-1 used $5x10^4$ cells per well, reporting a red pigment with an IC_{50} of 0.04, 0.2, and 0.55 μg/mL (in their respective fractions F5, F6, F7). 04, 0.2, and 0.55 μg/mL (in their separate fractions F5, F6, F7), another stain reported corresponds to *Streptomyces* sp. strain JAR6, which used a cell density of $1x10^4$, giving an IC_{50} of 145 μg/mL. This behavior that the IC_{50} decreases with increasing cell density has already been reported in the literature [103] There is a strong influence on the number of cells per well, which generates a reduction of cytotoxic activity [103].

The purification of the pigments is one of the critical parts for the bioactivities preliminarily tested to continue to have a significant effect [82], [104]. Since the cleansing of the colored *Streptomyces,* extracts are supposed to allow obtaining pure or more refined fractions that extract in a more enriched way the bioactive pigment that is considered the precursor of the antimicrobial, antioxidant, and cytotoxic results [82], [104].

As can be seen in Table 9, there is a summary of the *Streptomyces* pigments that report their respective yields. However, something that could be improved for future research in their papers is the specification of their products during the crude phase and the fractionated or purified grade. In this way, a global analysis can be made of how economically feasible these yields would be on an industrial scale. This would simplify the search for optimization factors since the economic factor would be considered, whether in the construction of the culture media or selection of solvents for extraction, among other factors to be considered [105]–[108].

An example is the extraction of the different pigments reported, as described in the results section. There is no standardized methodology since each molecule is a world of its own and has an infinite number of possible chemical configurations [109], [110]. It would be of great value to consider the preliminary performance of the raw pigment to see if it is attractive enough to scale up or lead to optimization so that research would focus more on the production of innovative pigments, that allow for simple fractionation with a possibility of high yields, and that can be easily scaled up both operationally and economically [111]–[113]. Since many of these pigments remain in the separation phase, some are very soluble, have unknown chemistry, and act as a black box, so very complex chromatography systems, which are somewhat expensive and difficult to recreate in the laboratory, must be used [114]–[117].

As described above, purification is a decisive factor in many aspects of validating the feasibility of a pigment [65], [67]. However, optimization is another factor to consider [118], [119]. Many independent and dependent variables can influence these secondary metabolites [42], [119], [120]. It has been mentioned that *Streptomyces* can produce their pigments due to their genetics, which may be unique to each species [121], [122]. Additionally, the composition of the collection source may affect that genetic configuration and, therefore, the conditions to which they are subjected in the laboratory [123]–[125].

A striking aspect of the results reported in Table 10 is that the optimized variables only present factors such as carbon source, temperature, pH, presence of salt traces, incubation time, nitrogen sources, and the effect of some minerals. But they do not report optimization in terms of agitation speed, which is one of the independent variables that most affect the production and yield of *Streptomyces* pigments [42], [118], [119]. It would be an aspect to analyze in-depth because then it means that many of the articles reviewed do not specify, whether these optimizations are being performed in solid medium or liquid fermentation or if only the factors mentioned initially are being changed, leaving the term agitation fixed within a standard range [15]. Additionally, it would be beneficial to say the type of experimental design being implemented [15]. This allows an analysis of how feasible it would be to apply one model to another if similar pigments or investigations following the same approach were being worked on.

2.7 CONCLUSIONS

The genus *Streptomyces* is recognized in the scientific world for its ability to generate all secondary metabolites, especially pigments that generally show perfect antimicrobial activity. This study proposed a literature review of articles reporting *Streptomyces* pigments. The most evaluated activities and those that showed positive or promising results were reviewed. The variables that could affect pigment yield, the types of extraction most applied to achieve adequate purification of the bioactive element, and general results that can give an insight into the direction that research on pigments produced by *Streptomyces* is taking.

According to what has been discussed and the results collected, it can be concluded that this review had a relevant scope, since it evidences the strengths and weaknesses that are being presented in *Streptomyces* pigment research worldwide. There is a substantial lack of academic contribution in continents such as Africa and South America. An essential scientific increase in this subject is observed on the Asian continent. On the other hand, there is strong scientific evidence of the bioactivities of these pigments. The most common ones are reported: antimicrobial, antioxidant, and cytotoxic. In addition, methodological standardization is lacking in evaluating antioxidant activity, which could be done globalized or with complementary assays.

It is important to note that very few articles report their yields and often do not describe in which phase this yield was determined, whether it was carried out in the crude or fractionated extract phase. This information would be helpful for a more in-depth analysis of the economic feasibility of producing pigments from *Streptomyces*. Finally, there is a lack of information regarding experimental designs to optimize the production of these pigments, with which it would be possible to outline when to apply an experimental design over others, what factors define the selection of this design, and what methodology could be used to select an adequate statistical model.

3 CHAPTER 3: BIOACTIVITY OF COLORED EXTRACTS FROM *Streptomyces* STRAINS WITH POTENTIAL COSMETIC APPLICATION

3.1 ABSTRACT

Personal care has become an essential part of our daily routine. Therefore, consumers today are looking for quality, safety, and innovation in the cosmetic products they buy. One of the most controversial components in cosmetics production is pigments and dyes. As previous studies have shown, these can trigger degenerative diseases and hormonal alterations in people's health. In addition, they could harm the environment. This research aimed to evaluate colored extracts from 19 *Streptomyces* strains isolated from Arauca and Guaviare riverbanks that can be converted into cosmetic ingredients with antimicrobial, antioxidant, and non-cytotoxic properties. Antimicrobial activity was performed using the disk diffusion technique. Cell viability was assayed in a 96-well plate with different cell lines such as HDFa and HeLa, and radical scavenging activities were evaluated by DPPH titration. The colored extract with the highest active potential was scaled to 1 liter of fermentation in ISP2 medium at appropriate conditions and extracted with ethyl acetate, yielding a fractionated pigment. The unrefined colored extract obtained from *Streptomyces* strains 145 and 290 showed inhibition against *B. subtilis*, *E. faecium, S. aureus*, and *S. epidermidis*. The percentage of free radical uptake was 22.1% and 5.21% for strains 145 and 290, respectively. Cell viability (CV), at the maximum concentration evaluated (1000 ug/mL) of strains 145 and 290, showed cytotoxic activity against HeLa cells, decreasing CV by 50-56.8%, respectively. The colored crude extract showed moderate antimicrobial, cytotoxic and low antioxidant activity at a concentration of 10 mg/mL, making it a potent cosmeceutical ingredient in the cosmetic field.

Keywords: *Streptomyces*, Pigment, Antioxidant, Antimicrobial, Cytotoxic, Cosmeceutical

3.2 INTRODUCTION

Personal care is an activity that has become essential to society [126], [127], and today consumers demand quality, safety, and innovation [128]. Cosmetics are substances or mixtures of ingredients with a specific purpose, benefit, or action on the skin [127]. Today, there are hundreds of cosmetic products distributed in different categories: skin care, hair care, decorative cosmetics, toiletries, and fragrances [129].

The cosmetics industry has a highly competitive market share because it penetrates different economic segments, from luxury and high-tech products to mass markets [129], [130]. Over the past 20 years, this industry has shown an average growth rate of 4.5% per year [131], which has allowed it to strengthen its sales [131], (it is expected to earn approximately US$ 429.8 million by 2022) [129]. The market size will grow at a compound average growth rate of 4.6% until 2025 [129], which will be valued at approximately US$ 33.8 billion [129]. The evolution of this industry has been shaped by several public interest factors, such as changing lifestyles, enhanced health awareness, use of natural ingredients, increasing GDP (Gross domestic product), development and innovation of new products, and government regulations [130]. Because of this, a new market has been incorporated into the personal care industry known as natural, and organic cosmetics [130], which consists of appropriating natural and organic ingredients in cosmetic formulations, and replacing the vast majority of synthetic ingredients or those that have adverse effects on human health [127], [130]–[132]

The current cosmetics have hundreds of substances and compounds that have been classified as dangerous to human health [133]–[135], among them, the most prominent are the colorants, for being one of the most used materials in this industry [135], [136], whose purpose is to develop decorative formulas such as lipsticks, makeup, eyeliners, shadows, etc. and enhance the product, making it more attractive in terms of marketing [1], [135]. However, these substances are generators of free radicals or have a high load of heavy metals, being the main precursors of metabolic disorders and genetically influencing the body to develop immune diseases such as arthritis, cancer or accelerate premature aging of the body [2], [133], [135]. In addition, there is a high environmental impact involved in the production of these substances [3]. It is estimated that around 100,000 dyes and pigments are marketed today, representing 700,000 tons of production, reflecting 10-15% of the effluent contamination [3].

Natural pigments are an alternative to organic and inorganic pigments of synthetic origin [2], [137]; they are generally obtained from seeds, plants, fruits and roots, and are strongly dependent on variables such as climate, soil types, cell tissue types, and seasonality, among others [137]–[139]. This makes current production methods economically unviable for the food, cosmetic and pharmaceutical sectors, significantly increasing the product's value in the market and its availability [137]–[139]. In accordance with this, research has been carried out on the production of natural pigments from microorganisms [138], since the production process is more feasible in economic terms and the production of the

pigment does not depend on climatic, topographical, or cellular conditions, but on the manipulation of cultivation or genetic variables [138]. Nowadays, there are several patents on this subject, of great interest at industrial level, three of them are mentioned here: 1) Patent No. 9493792, the production of a yellow pigment from *Bacillus* sp. GSK07 containing D-limonene and soluble in different polar solvents, its use has been determined for the food, cosmetic and pharmaceutical industry as a colorant, flavorant or fragrance and in addition to that it has a good antioxidant activity [140]. 2) Patent No. CN20061034149 20060307 refers to a new strain of *Streptomyces* called *Streptomyces vietnamensis* producing the purple blue pigment with high UV stability, thermal and high temperature resistance, a prospect of great potential to be developed as a natural non-toxic pigment [50], [141]. 3) Patent No. 5814495, Melanin production from *Streptomyces* with modifications in growth medium, fermentation conditions and application of genetic engineering [141].

The *Streptomyces* is one of the most representative genera of the class of Actinobacteria or Actinomycetes [142], [143]; its structure corresponds to an aerial mycelium and with branches very similar to the structure of fungi, it represents 50% of the population of soil actinomycetes [54], its genome contains more than 55% of cytosine and guanine, which allows it a facility when making genetic modifications in its DNA [144], produces about 80% of the actinobacterial products that exist today [17], this microorganism plays a very important role in the biosynthesis of secondary metabolites, especially in the production of pesticides, antibiotics, biotechnological enzymes [17], [57], [83], and as mentioned above in the production of pigments [45], [46]. In previous studies, it has been reported that many of these pigments present beneficial biological activities for humans [45], [46], [145], however, their application has been more limited to the food or textile industry [54], [76], [83], [136], [137].

Today, the cosmetics industry talks about the term cosmeceutical [146], which is widely applied in one of its most important categories: the skin care category [97], [147]–[149]. Due to its positive economic impact on sales [130], [149], the products in this category have become the most demanded by users [130], [149]. Cosmeceuticals are biological active ingredients that have therapeutic or medicinal properties on the skin [128], [146], this has allowed the development of high-tech dermocosmetic formulations with many benefits for consumers [147]. These benefits are based on many biological activities, the most common are: antimicrobial [150], antioxidant [15], [151] and cytotoxic [151], since they are the most evaluated in the industry, because the conditions for an ingredient to be incorporated into a product is the safety and quality that this represents to the consumer [127], [152], should not generate adverse effects, or alter the cellular nature of the body, or represent a long-term toxic buildup [98]. Therefore, the purpose of this study is to evaluate the bioactivity of colored extracts produced by *Streptomyces* with potential cosmetic application, becoming a possible cosmeceutical ingredient that can be incorporated in the next generations of cosmetic formulas from natural origin [97], [127].

3.3 METHODOLOGY

3.3.1 Strains, media, and culture conditions

La Universidad de La Sabana has a bioprospecting research group that has a bank of Actinobacteria obtained from sediments of the Arauca and Guaviare rivers with a morphology like that of *Streptomyces*, which have been cultivated and preserved in ISP2 medium (g/L composition: Malt extract, glucose, yeast extract and agar) and ISP3 medium (g/L composition: Oats, trace salt solution and agar) [153], [154]. Nineteen strains from both rivers were selected from this bank [153], [154]. The selection criteria are based on previous studies reported by N. Pastrana and C. Arango, where it was observed that these strains strongly pigmented the culture medium (ISP2- ISP3) [153], [154]. The selected strains were thawed and reactivated in the same medium in which they were cryo-concentrated, (ISP2-ISP3) at -70°C in a Revco (Thermo Scientific™, USA) high performance freezer [29]. After sowing the spores of each strain on agar medium, they were incubated for 7 days at 30° C [15], [45].

After incubation, a section of $1\ cm^2$ was cut to suspend it in 10 mL of the same medium (ISP2-ISP3) but liquid, and it was left another 7 days at 30 ° C with constant agitation (150 rpm), each experiment was done in triplicate [15], [45]. At the end of the incubation time, the *Streptomyces* biomass from the pigmented medium was centrifuged, subjected to lyophilization to make the extraction of the respective pigment, to evaluate their bioactivities [155].

3.3.2 Colored extract extraction

The pigments were extracted as follows: Pigmented media and biomass were centrifuged at 5000 x g for 10 min (Sorvall® Series Centrifuge ST16, Thermo Scientific™, USA) to separate them, both products were lyophilized as mentioned above, using the Freezone® 2.5-liter Benchtop Freeze Dry equipment (Labconco, USA) from the bioprocess laboratory of La Universidad de la Sabana. The dry colored extracts obtained from the medium were redissolved in 10 ml of water:ethanol (70:30) for its subsequent evaluation of bioactivities. [47].

3.3.3 Antibacterial activity

The antibacterial activity was evaluated by means of the disc diffusion technique against Gram-negative and Gram-positive bacterial strains: *Escherichia coli* (ATCC® BAA-469), *Escherichia coli* (ATCC® BAA-2469), *Escherichia coli* (ATCC® BAA-2649), *Klebsiella pneumoniae* (ATCC® 700603), *Klebsiella pneumoniae* (ATCC® 11486), *Pseudomonas aureginosa* (ATCC® 27853), *Bacillus subtilis* (ATCC® 21556), *Staphylococcus aureus-MRSA* (ATCC® BAA-44), *Staphylococcus epidermidis* (ATCC® 12228), and *Enterococcus faecium*- VRE (ATCC® 700221) [156]. The paper disks were impregnated at different concentrations 10, 15 and 30 mg/mL in various volumes (10, 15, 30 mL) of the extracted pigments in the appropriate solvent [155]. These discs were placed in plates

with Muller-Hilton agar (Sigma Aldrich), previously inoculated with the pathogenic strains [155]. The plates were incubated for 24 h at 37° C, and the bioactivity of the pigments was determined by measuring the inhibition zone [155]. The diameter of the inhibition halos (Dh) was measured in mm and established as follows: Dh <10 mm: + low activity, Dh 10-20 mm: ++ moderate activity and Dh >20: +++ high activity. The experiment was carried out in duplicated, and the inhibition zones were expressed as mean ± SD (standard deviation) [153].

3.3.4 Antioxidant activity

The antioxidant activity of the crude extracted pigments was analyzed using the DPPH (2,2-diphenyl-1-picrylhydrate, Sigma Aldrich, USA) method [46], [157], [158]. The test was performed in a 96-well microtitre plate (iMark™ , Bio-Rad, USA).). Each crude extracted pigment was taken at 5 and 10 mg/ml and mixed with 100 μL of DPPH, leaving them in the dark for 30 min, the reduction of free radicals was determined by measuring the absorbance of the samples at 517 nm, and the absorption of the free radicals in the colorless DPPH was expressed in percent, as shown in equation 1, A_S is the absorbance of the sample (pigment) and A_DPPH, the absorbance of the DPPH solution [46], [157], [158]. Vitamin C was used as a positive control. Its curve was performed with the following concentrations 100mM, 50mM, 25mM, 12,5mM, 6,25mM, 3,125mM and 1,5625mM. All tests were performed in triplicate.

Equation 1

$$\%RSA = (\frac{A_{DPPH} - A_S}{A_{DPPH}})/100$$

3.3.5 Cytotoxic Activity

Cell viability was determined with human fibroblasts (ATCC® PCS-201-012™) Cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum and 100 mg/mL penicillin [15]. The cells were kept under 97% humidity in a biological incubator (Bio-rad , USA) at 37°C with 5% CO2 [159]. 100 μL of cell suspension was inoculated into 96-well plates with a density of $5x10^3$ cells/well and left for 24 h [159]. After this time, the culture medium was replaced with 100 μL of serum-free medium containing different concentrations of crude extracted pigment (1000-3.95 μg/mL) [159]. The test was carried out in triplicate. DMSO at 3% (1-25 ppm) was used as the positive control, the water/ethanol solvent mixture (70:30), with which the crude extract was obtained, was used as a negative control (100% survival), and DMEM medium was used as the target [159].

The MTT method was used, following the methodology described by K. Krishnan and others with some modifications to determine cell cytotoxicity [151]. 10 μL of MTT was added to a concentration of 5 mg/mL per well and were left to incubate for 48 h at 37ºC

and 5% of CO_2 [40]. After this time, the formazan crystals were dissolved with 100 µL of DMSO, absorbance was read at 570 nm in a microplate reader [151]. Cellular cytotoxicity was expressed as percentage of viability (equation 2) and reported as mean IC_{50} (maximum mean inhibitory concentration) ± SD [151].

Equation 2

$$\% \, viability = (\frac{Number\ of\ live\ cells}{Total\ number\ of\ cells})x10$$

All materials and reagents used for cell culture were purchased from Sigma Aldrich (USA).

3.3.6 TLC of crude extract and fractionation of active compound

Separation of antimicrobial compound from the crude extract was done by thin layer chromatography (TLC), using silica gel coated chromate plates (60G F254, Merck, Germany) [47]. The best solvent system was determined by making the following combinations: ethanol and ethyl acetate (Sigma Aldrich) (60:40, 50:50, 70:30, 30:70, 80:20, 20:80, 0:100) and the mixture reporting the Rf value (retention factor, equation 1) with a better separation of the compound was chosen, using equation 3 [2]. The solvent system was allowed to run, a TLC plate not impregnated with sample was used as a control target, once the solvent system was completed the TLC plates were allowed to air dry and observed in a UV light chamber (Merck, Germany) at 254 and 365 nm and in the visible one before and after treatment with developing agents [2], [153].

Equation 3

$$R_f = \frac{Distance\ travelled\ by\ the\ soluto}{Distance\ travelled\ by\ the\ solvent}$$

The crude extract was therefore extracted with pure ethyl acetate. The supernatant and solvent were shaken vigorously for 30 min, and then was allowed to stand for another 15 min, to form the separation of the aqueous phase from the organic phase, then it was left to decant until the bioactive compound (pigment) was separated. It was collected and concentrated in a rotary vacuum evaporator (Hei-VAP Expert, German) [2], [160]

3.3.7 Antibacterial activity of the fractionated extract

Based on the results of the bioactivities with the crude extract, the strain with the best antibacterial and cytotoxic behavior against cancer cells was selected. The assay was performed bioguided, that is, once the crude extract was fractionated [47], [161], its antibacterial activity against *S. epidermidis* was evaluated using disk diffusion techniques, evaluating a single concentration (30 mg/mL), following the MacFarland scale at 0.5% to establish the concentration of the bacteria, and taking Vancomycin as a positive control,

and the solvent used to dissolve the fractionated extract (ethanol 30: Water 70) as a negative control [47].

Additionally, the growth of *S. epidermidis* was monitored using the Bioscreen C analyzer (Growth Curve USA®), under aerobic conditions, 100 microliters of bacteria ($1x10^6$CFU/mL) were inoculated in each well along with different concentrations of the fractionated extract, 300, 30, and 3 ppm, the negative control was the vehicle (ethanol 30: water 70), positive control Gentamicin at 40, 4 and 0. 4 mg/mL, incubating the HoneyComb type plate at 37 degrees, with a reading time of 24 hours, at 30-minute intervals, at an optical density of 600 nm [162].

3.4 RESULTS AND DISCUSSION

3.4.1 Strain selection and colored extract extraction

Twenty strains were evaluated and reactivated in their respective cryopreservation media (ISP2 & ISP3) on solid medium. Each of the strains that gave pigmentation in a solid medium was then scaled in both liquid media (ISP2&ISP3) to observe pronounced pigmentation changes. Of the 20 strains evaluated, only 3 strains showed intense pigmentation between a range of yellow to orange in liquid medium (ISP2) as seen in Figure 1; each fermentation was done in duplicate. Modification of carbon and nitrogen sources and other operational factors have revealed relevant results in melanin production by El-Naggar et al. (2017), one of the pigments most produced by *Streptomyces* [15].

*Figure 6 Production of the three colored fermentations in ISP2 liquid medium from strains 145, 627, and 290. ISP2 medium, without inoculum, was used as a control indicator. * The number following the strain indicates the code of the agar batch from which the inoculum was taken for seeding in liquid.*

3.4.2 Antimicrobial activity results

The antimicrobial activity (AM) was evaluated against 10 pathogenic bacteria. The colored extracts 145 and 290 showed moderate AM against *B. subtillis, E. faecium, S. aureus and S. epidermidis*. The colored extract 627 showed no antimicrobial effect. The antimicrobial activity was determined quantitatively, by measuring the inhibition halos, as summarized in Table 11 for each extract.

Figure 7 compiles the results corresponding to the antimicrobial behavior of the colored extract 145. It is observed that at the global concentrations of 10 and 15 mg/mL for B. *subtillis*, an inhibition halo of 10 mm is obtained, at the final concentration in the disc of 0.3 and 0.45 mg, and at the maximum global concentration of 30 mg/mL, in the three different final concentrations (0.3, 0.45 and 0.9 mg), there is no significant change at the concentrations previously evaluated.

As for *E. faecium*, the colored extract 145 showed a superior AM compared to the nalidixic acid control, which presented an inhibition halo of 9.0 ± 0.0. At concentrations of 10 and 15 mg/mL in the highest volumes of 15 and 30 μL, inhibition halos of 9.5, 10, and 12 mm were obtained.

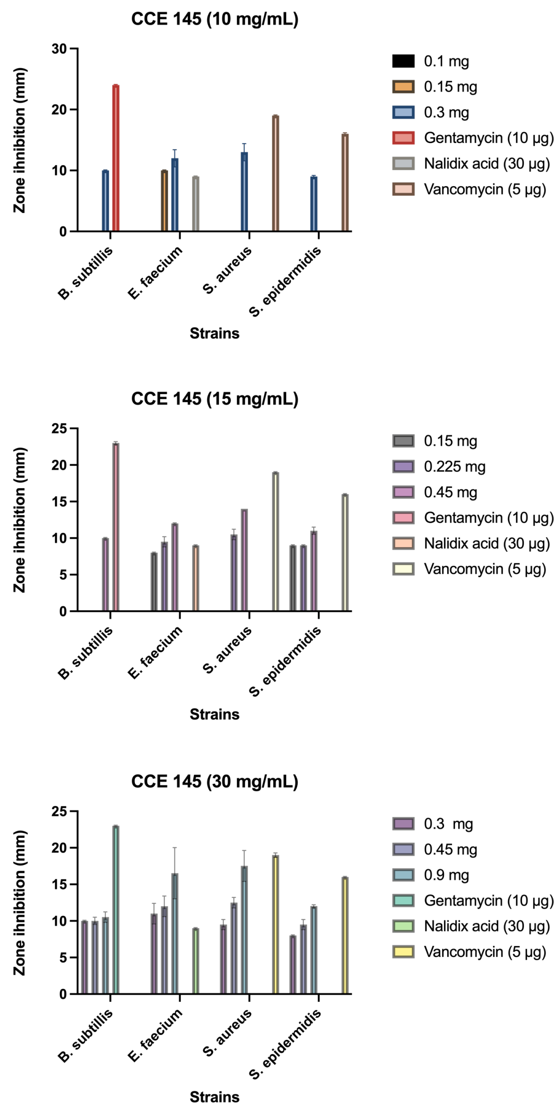


Figure 7 Diameter of the zones of inhibition by disk diffusion. Three global concentrations were evaluated for the colored crude extract of Streptomyces strain 145 (10, 15, and 30 mg/mL), with their respective final concentrations at which the discs were left. It was used as a positive control for B. subtillis (Gentamycin), E. faecium (Nalidixic acid), S. aureus, and S. epidermidis (Vancomycin). Negative control (30 uL) water:ethanol (70/30 v/v). The bars do not appear because they did not present any typed of inhibition.

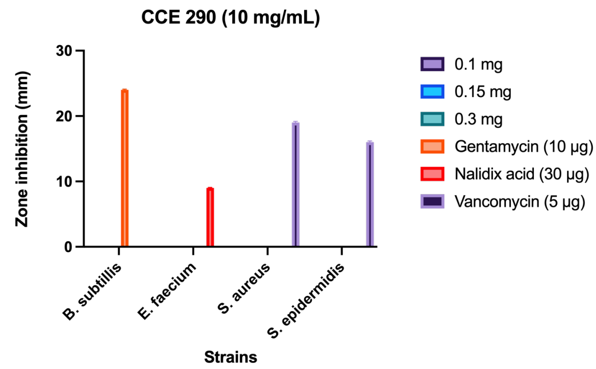
Table 11 Antibacterial efficacy of Streptomyces colored extracts 145 and 290 (obtained at different concentrations 10, 15, 30 mg/mL and volumes in the disc (10, 15 and 30 μL) against Gram-positive and Gram-negative bacteria.

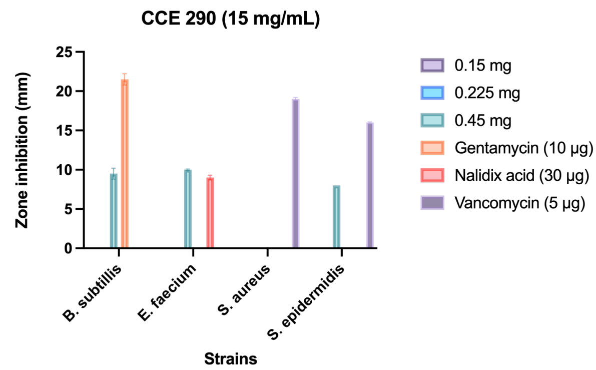
Extract	Concentraction mg/mL	Inoculated volume in the sensidisc (μL)	Halo (mm)	Control (+)	Halo, control + (mm)	Bacteria
145	10	10	-	Gentamycin (10 μg)	24 ± 0.1	*Bacillus subtilis*
		15	-			
		30	10 ± 0.1			
	15	10	-		23± 0.2	
		15	-			
		30	10 ± 0.1			
	30	10	10 ± 0.1		23± 0.1	
		15	10 ± 0.5			
		30	10.5 ± 0.71			
	10	10	-	Nalidixic acid (30 μg)	9.0 ± 0.1	*E. faecium*
		15	10 ± 0.1			
		30	12 ± 1.4			
	15	10	8.0 ± 0.1		9.0 ± 0.1	
		15	9.5 ± 0.7			
		30	12 ± 0.1			
	30	10	11 ± 1.4		9.0 ± 0.1	
		15	12 ± 1.4			
		30	16.5 ± 3.5			
	10	10	-	Vancomycin (5 μg)	19.0 ± 0.1	*S. aureus*
		15	-			
		30	13 ± 1.4			
	15	10	-		19.0 ± 0.1	
		15	10.5 ± 0.71			
		30	14 ± 0.0			
	30	10	9.5 ± 0.7		19.0 ± 0.3	
		15	12.5 ± 0.71			
		30	17.5 ± 2.12			
	10	10	-	Vancomycin (5 μg)	16.0 ± 0.2	*S. epidermidis*
		15	-			
		30	9.0 ± 0.2			
	15	10	8.0 ± 0.1		16.0 ± 0.1	
		15	9.0 ± 0.1			
		30	11 ± 0.5			
	30	10	8.0 ± 0.1		16.0 ± 0.1	
		15	9.5 ± 0.7			

		30	12 ± 0.2			
Control (-) Ethanol 30: Water 70 (30 µL)			* Negative			

290	10	10	-	Gentamicyn (10 µg)	24 ± 0.1	*Bacillus subtilis*
		15	-			
		30	-			
	15	10	-		21.5± 0.71	
		15	-			
		30	9.5 ± 0.7			
	30	10	-		23± 0.1	
		15	9.5 ± 0.7			
		30	10.5 ± 0.71			
	10	10	-	Nalidixic acid (30 µg)	9.0 ± 0.1	*E. faecium*
		15	-			
		30	-			
	15	10	-		9.0 ± 0.3	
		15	-			
		30	10 ± 0.1			
	30	10	-		9.0 ± 0.1	
		15	9.5 ± 0.7			
		30	13 ± 1.4			
	10	10	-	Vancomycin (5 µg)	19.0 ± 0.2	*S. aureus*
		15	-			
		30	-			
	15	10	-		19.0 ± 0.1	
		15	-			
		30	-			
	30	10	-		19.0 ± 0.1	
		15	-			
		30	14.5 ± 0.71			
	10	10	-	Vancomicina (5 µg)	16.0 ± 0.2	*S. epidermidis*
		15	-			
		30	-			
	15	10	-		16.0 ± 0.1	
		15	-			

		30	8.0 ± 0.1			
	30	10	-		16.0 ± 0.1	
		15	9.0 ± 0.1			
		30	10 ± 0.1			





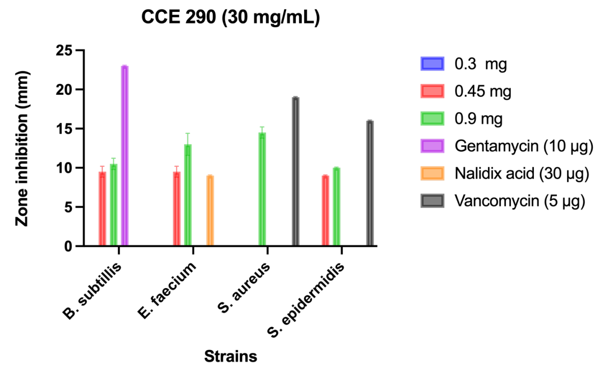


Figure 8 Diameter of the zones of inhibition by disk diffusion. Three global concentrations were evaluated for the colored crude extract of Streptomyces strain 290 (10, 15, and 30 mg/mL), with their respective final concentrations at which the discs were left. It was used as a positive control for B. subtillis (Gentamycin), E. faecium (Nalidixic acid), S. aureus, and S. epidermidis (Vancomycin). Negative control (30 uL) water:ethanol (70/30 v/v). The bars do not appear because they did not present any typed of inhibition.

In Figure 8, the behavior of the colored extract of *Streptomyces* 290 strain at the final concentrations in the disk can be observed. In the case of *B. subtillis*, inhibition was only presented at a concentration of 0.45 and 0.9 mg (overall concentrations of 15 and 30 mg/ml), indicating an antimicrobial potential concerning the control. As for *S. aureus*, only at the highest final concentration of 0.9 mg, inhibition was very similar to that of the vancomycin control.

Figure 9 shows the behavior of the inhibition halos of extract 145 at a concentration of 30 mg/mL against *S. aureus* and *S. epidermidis*, which presented the best antimicrobial activity of the three extracts evaluated.

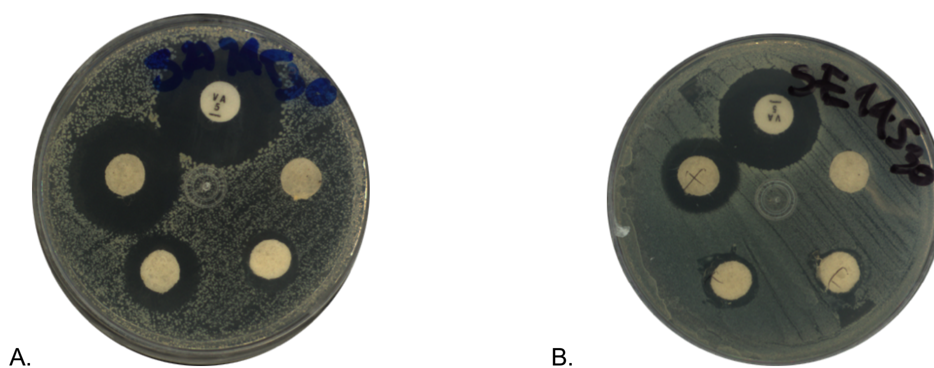


Figure 9: ***Antimicrobial activity of crude extracts of strain 145 by disk diffusion technique****. A) Concentration evaluated of raw colored extract 145 to 30 mg/mL in S. aureus. Vancomycin (positive control), 70% water/ 30% ethanol (negative control). Volumes tested: 10 µL (10-9 mm), 15 µL (13-12 mm), and 30 µL (19-16 mm). B) Concentration evaluated of raw colored extract 145 to 30 mg/mL in S. epidermidis. Vancomycin (positive control), 70%water/30% ethanol (negative control). Volumes tested: 10 µL (8 mm), 15 µL (9-10 mm) and 30 µL (19-16 mm).*

3.4.3 Antioxidant activity results

The crude extracts of strains 145 and 627 showed relevant antioxidant activity at 5 mg/mL; doubling the concentration (10 mg/mL) increased their free radical scavenging capacity, as shown in Table 12. Extract 290 showed a very minimal percentage of free radical uptake at 5 mg/mL; however, upon doubling its concentration, it showed 5.26% of SCV, a similar effect to that achieved with vitamin C at a concentration of 3.125 mM.

*Table 12 **The antioxidant activity demonstrated by the colored extracts of streptomyces**. Results are presented as means ± SD. The colored extracts correspond to the crude extract (Unrefined), extracted from the supernatant with the solvent water:ethanol (70/30 v/v). Each treatment was carried out in triplicate (p<0.05). (*) % SVC = radical scavenging activity. All colored extracts were measured at a concentration of 10 mg/mL.*

Colored Extract	%SCV (*)
145	22.06 ± 0.0021
290	5.27 ± 0.0015
627	53.73 ± 0.0074

3.4.4 Cytotoxic Activity Results

The crude extracted pigments were tested in human fibroblast, and Hela cells, the results from IC_{50} varied for each crude colored extract (CCE), however, one of the extracts showed no cytotoxicity (Table 3). When comparing IC_{50} shown in Table 13, colored extract 627 has no cytotoxicity, however, CCE 145 and 290 have high to moderate cytotoxic activity (CA), respectively, regarding the DMSO, which is considered an inducer of apoptosis at high concentrations [54]. CCE 627 had the lowest percentage of dead cells (102%) at the maximum concentration of 1000 µg/mL.

*Table 13 **IC50 (µg/mL) of the colored crude extracts of three Streptomyces** sp. strains **in human fibroblast cells.** Results are presented as means ± SD. Each treatment was carried out in triplicate (p<0.05). (*) HDFa= Human Dermal Fibroblast.*

Crude color extract	IC50 µg/mL	Cytotoxic activity
	HDFa (*)	
145	25.11 ± 7.074	High
290	67.07 ± 3.88	Moderate
627	-	No cytotoxic

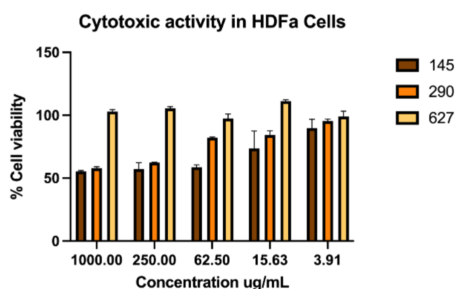


Figure 10 cell viability of colored extracts of Streptomyces sp. in HDFa cells. Each treatment was carried out in triplicate (p<0.05).

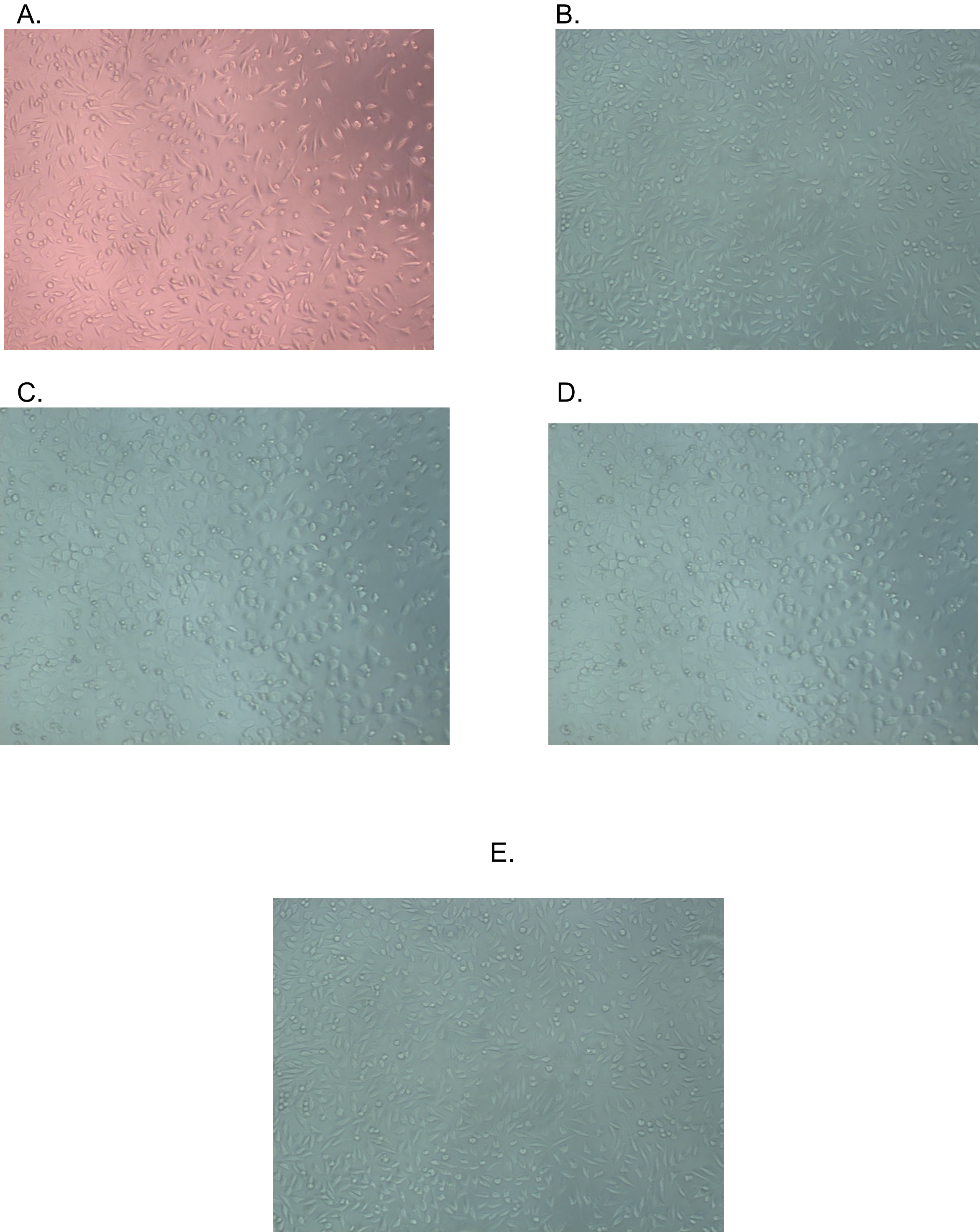


Figure 11: ***Cell viability in HDFa (Human dermal fibroblast) cells when treated with the colored extracts of strains 145, 627, and 290.*** *A) HDFa cells + Vehicle (Water 70 | Ethanol 30), 100% cell viability. B) The appearance of HDFa + DMSO 10% cells (Well F1). Cell stress retracted cell membrane without projections can be observed—cell viability 8.49%. C) The appearance of HDFa cells that were inoculated with 1000µgmL-1 of extract #145 (Well A1). Cell stress can be observed—cell viability 54.77%. D) The appearance of HDFa cells that were inoculated with 1000 µgmL-1 of extract #290 (Well A4). Cell stress can be observed—cell viability 57%. E) The appearance of HDFa cells that were inoculated with 1000µgmL-1 of extract #627 (Well A7). No cell stress. Cell viability 100%.*

Table 14 IC_{50} (µg/mL) of the colored crude extracts of Streptomyces sp. *in Hela cells. Results are presented as means ± SD. Each treatment was carried out in triplicate (p<0.05).*

Crude color extract	$IC50$ µg /mL HeLa Cells	Cytotoxic activity
145	37.34 ± 2.90	High
290	93.21 ± 1.76	Moderate
627	-	No cytotoxic

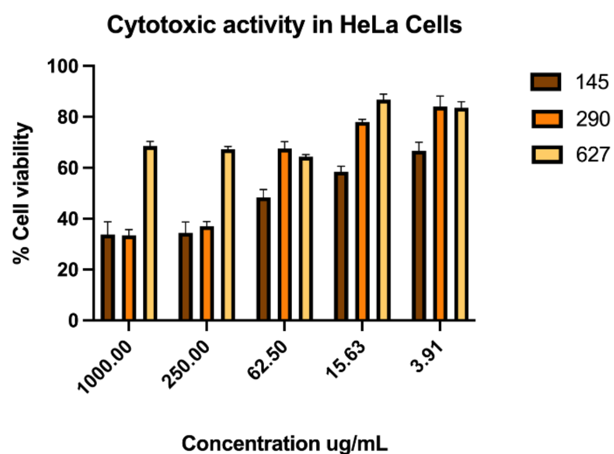
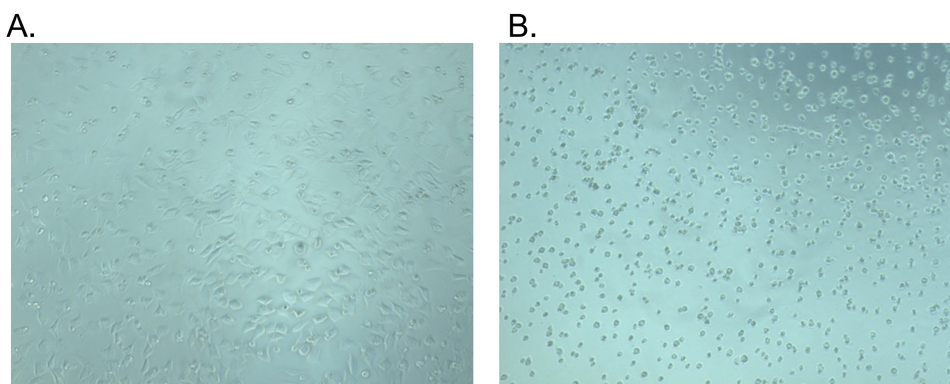


Figure 12 cell viability of colored extracts of Streptomyces sp. *in HeLa cells. Each treatment was carried out in triplicate (p<0.05).*



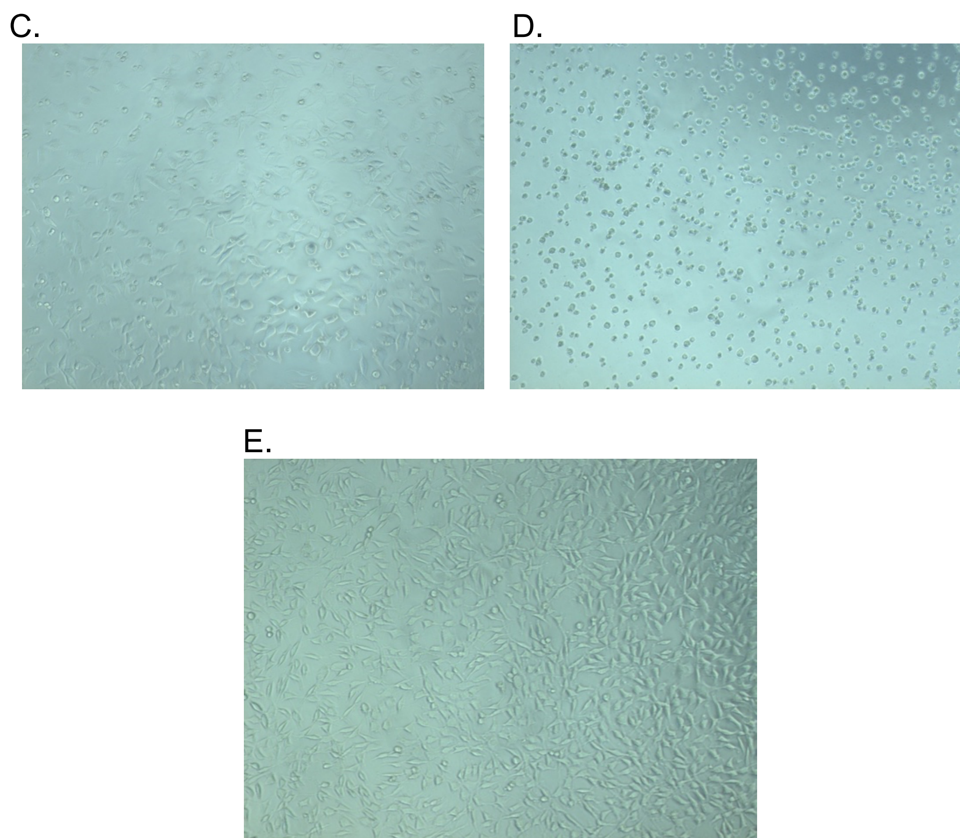


Figure 13: ***Cell viability in HeLa cells when treated with the colored extracts of strains 145, 627, and 290.*** *A) The appearance of HeLa cells + Vehicle (Well F9). Cell viability is 98%. B) The appearance of HeLa cells + DMSO 10% (Well F1). Cell stress retracted cell membrane without projections, cell viability 8.36%. C) The appearance of Inoculated HeLa cells with 1000 µgmL-1 of extract #145 (Well A1). Cell stress can be observed—cell viability is 36.6%. D) The appearance of Inoculated HeLa cells with 1000 µgmL-1 of extract #290 (Well A4). Cell stress can be observed—cell viability is 36.25%. E) The appearance of Inoculated HeLa cells with 1000µgmL-1 of extract #627 (Well A7). No cell stress. Cell viability is 62.2%.*

3.4.5 TLC of crude-extract and fractionation of active compound

The pigment fermentation was carried out in an incubation time of 7 days, at normal agitation and temperature conditions (100 rpm and 35 C, respectively). The culture broth used was ISP2 Universal, without any modification. The crude colored extract was obtained, separating the biomass from the supernatant by centrifugal forces, which was subsequently treated with a mixture of ethanol-water (30:70) v/v. The crude extracts were used in this way to achieve fractionation with the different solvent systems to find which generated the best separation.

The TCL profile of the bioactive enriched fraction of the colored extracts had a better separation with 100% ethyl acetate. Using this mobile phase, the crude extract was run on a silica plate, obtaining Rf with the following values for each of the crude extracts

obtained: 0.58 (Ext 290), 0.48 (Ext 145), 0.56 (627). A single-colored fraction was obtained for each pigment extract and was used to evaluate the subsequent bioactivities.

3.4.6 Results antibacterial activity fractionated extract

The results of the antimicrobial activity by disc diffusion technique can be seen in Figure 14, where each replicate shows a positive inhibition of *S. epidermidis* bacteria when exposed to fractionated extract 145 at a concentration of 30 mg/mL, in contrast with the commercial antibiotic vancomycin 5 μg.

The growth rate was calculated using a curve fitting method in Combase software, whose proposed model corresponds to a linear model for 145 fraction extract and commercial antibiotic develop with a R-square of 0.888 and 0.71 respectively. As summarize Table 15.

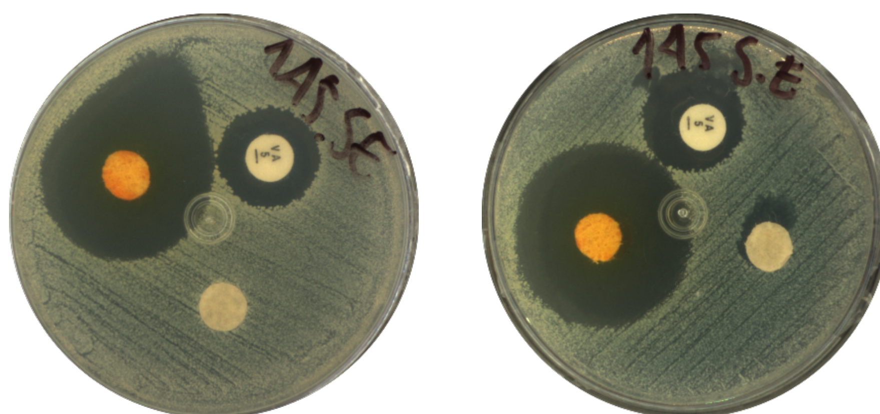


Figure 14 ***Concentration evaluated of fractionated extract 145 at 30 mg/mL in S. epidermidis****. * (Fractionated extract refers to the fraction extracted with 100% ethyl acetate). Vancomycin (positive control), 70% water/30% ethanol (negative control). The tested volume of the fraction was 30uL. Replicate 1: inhibition halo (17.8 mm), Vancomycin (8 mm). Replicate 2: inhibition halo (14.5 mm), Vancomycin (8 mm).*

Table 15 ***Growth rate and model fit of the fractionated extract of strain 145****, evaluated at three concentrations 0.4, 4 and 40 mg/mL. Each assay was performed in duplicate with p value < 0.05.*

Agents	Maximun Rate (logCFU/g)	Model fit
Gentamicine (40 mg/mL)	0.0019 ± 0.000661	Linear model
Gentamicine (4 mg/mL)	0.00209 ± 0.000747	Linear model
Gentamicine 0.4 mg/mL	0.00149 ± 0.000711	Linear model
Ethanol: water (30:70)	0.0973 ± 0.00342	Baranyi and Roberts

145 fraction extract (0.3 mg/mL)	0.00917 ± 0.000749	Linear model

The graph illustrated in Figure 15, shows the behavior of *S. epidermidis* against the positive control Gentamicin at different concentrations, the negative control ethanol: water, and the fractionated extract at 300, 30, and 3 ppm. It can be observed that extract 145, at 300 ppm, presents a very similar curve with the commercial antibiotic, reaching a percentage of microbial inhibition of 91% concerning the positive control that ranges between 96-98% inhibition, which is corroborated in Table 15 when analyzing the maximum rates calculated by the free Combase software.

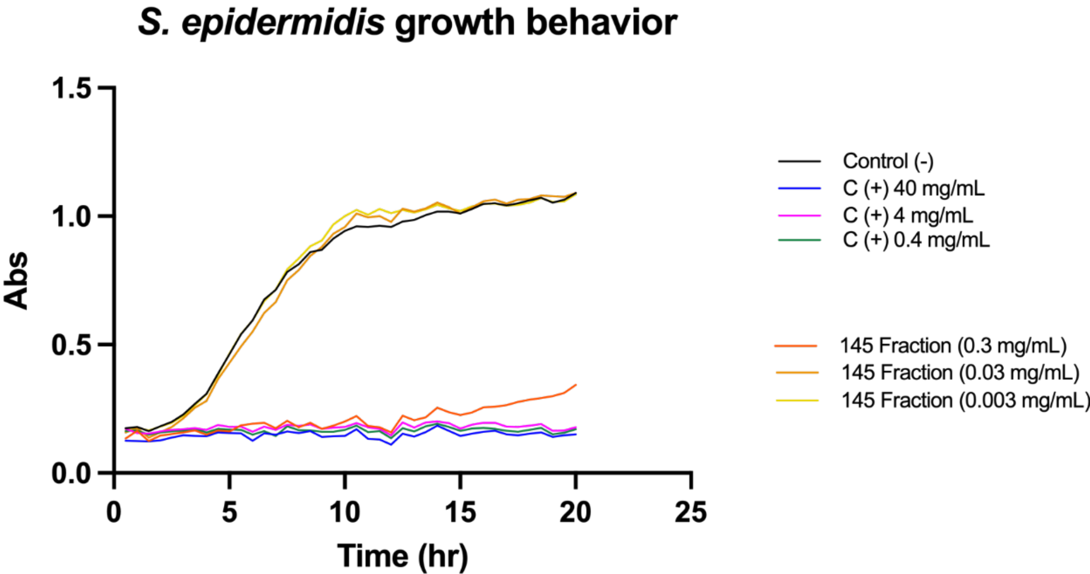


Figure 15 ***Growth curve of S. epidermidis, when treated with the ethyl acetate fraction of Streptomyces strain 145*** *evaluated, at three concentrations, 0.3, 0.03, and 0.003 µg/mL. The negative control corresponds to water: ethanol (70/30 v/v), positive control gentamicin at 40, 4, and 0.4 mg/mL. Each treatment was performed in duplicate with a p-value < 0.05.*

3.5 DISCUSSION

The *Streptomyces* genus is the most important source of bioactive molecules from actinomycetes. In recent years they have generated biotechnological applications of great utility for humans and the environment worldwide [35].

One of their most representative qualities is their antimicrobial potential against different pathogenic microorganisms. The investigations of Paranee S. et al. reported an actinomycete isolated from soil with grayish-yellow coloration in liquid medium (ISP2),

which showed AM against *B. subtilis* with an inhibition halo of 12.7 ± 0.58 mm [163]. On the other hand, Jayanthi et al. reported a red pigment from *Streptomyces sp*. JAR6, extracted with ethyl acetate, at a concentration of 50 mg/mL, using volumes of 25, 50, 75 and 100 μL in each disc, with inhibition halos (mm) of 16.23 ± 0.205 (25 μl), and 20.48 ± 0.139 (100 μL)[54].

Contrasting the data obtained from extracts 145 and 290 in this research with those previously described, we can highlight that the results presented are of great interest since it is observed that, at a lower concentration and volume dosage, a moderate antimicrobial behavior is generated against *B. subtilis*, very similar to that of the red pigment of *Streptomyces sp*. JAR6, however, there is no bioactivity against S. aureus. However, it should be remembered that the extract with which the disc diffusion assay was developed in this investigation consisted of crude ethanol|water (30:70) extract, without any purification or refinement treatment, as compared to that reported by Jayanthi et al [54].

In another investigation, Mariana Girao et al. [164] strain KENR94, identified with a pale-yellow extracellular coloration, said between 10-20 mm of inhibition halo against S. aureus [164]. This bacterium has substantial health implications for humans. A similar result was obtained, as shown in Table 11, where the extract 145 reaches inhibition halos of 10 to 17.5 (mm); this presumes a range of possibilities in the long-term sustainable obtaining of complementary components or new molecules from the Colombian Biodiversity [12], about the *Streptomyces* Biobank that was used in this research. Although the extract reported by Mariana Girao et al [164]. is indeed purified and, therefore, at a lower concentration (1000 μg/mL), this allows us to visualize that the crude extracts used for the development of this experiment contain molecules with potent antimicrobial activity, whose characteristics and functionalities can be increased or improved when the extract is fractionated [67].

Finally, another exciting result is compared against *S. epidermidis* on 7 *Streptomyces* strains isolated from the Actinomycetes Germplasm Conservation Bank of the Southeast Unit of CIATEJ [165], reporting inhibition halos between 12-31 mm. This broadens the possibility of the future development of a possible cosmeceutical makeup due to the pigmented nature of the extract obtained, which makes it very attractive, as it shows a positive antimicrobial activity against *S. epidermidis*.

Vitamin C is an excellent water-soluble antioxidant because it can donate hydrogen atoms and form stable free radicals [166]. Free radicals can accumulate in the body in unstable conditions, generating oxidative stress and triggering many diseases, including cancer [166]. Many *streptomyces* pigments have been reported as potential antioxidants. Dong-Ryung Lee et al [167] found that the culture supernatant of strain MJM 10778, subjected to filtration and double extraction with ethyl acetate and subsequently dried under vacuum, showed excellent antioxidant activity with increasing extract concentration (15 - 1000 μg/mL), reporting free radical uptake of 82.6% and 81.2% for 500 and 1000 ug/mL, respectively [167].

Although the uptake by crude extracts is low compared to the results reported by Dong-Ryun Lee [167], it should be noted that this assay was analyzed under an enriched fraction, which was subjected to a double extraction, to reach its respective elucidation, Therefore, it can be speculated that by purifying extracts 145 and 627, their capacity to capture free radicals could be potentiated, reaching concentrations lower than 10 mg/mL, and also have the advantage, compared to strain MJM 10778, that the extracts would have a coloration, being useful in the cosmetic industry.

In another study, they reported free radical uptake between 5.8% to 22.03%, in a concentration range of 0.5 mg/mL to 4 mg/mL and checked that the antioxidant capacity increases when increasing the concentration to 10 mg/mL, as observed with extracts 145, 290 and 627, the possibility of creating synergistic mixtures of extracts can be considered, enhance the antioxidant activity and thus be able to use a lower concentration and with greater efficacy [168].

Based on the results obtained from the *Streptomyces* CCE, it can be observed that extract 145 has better cytotoxic performance in HeLa cells compared to 290 and 627, receiving an IC_{50} of 37.34 μg/mL. In contrast with the literature reviewed during this experimentation, there is knowledge of cytotoxic activity against HepG2 cell lines by a crude extract of *Streptomyces parvulus* with a reported IC_{50} of 500 μg/mL, which is 13 times higher in concentration than that reported with strain 145 [169]. A similar result was declared by K. Saraswathis et al. with an ethyl acetate extract from *Streptomyces cangkringensis,* where the IC_{50} is 410.5 μg/mL, the determination of this anticancer activity was evaluated in a concentration range from 100 to 1000 μg/mL in HeLa Cells [170].

On the other hand, other studies report IC_{50} against non-tumor cells, such as HEK 293, of 64.11 μg/mL, very close to that obtained with crude extract 290. The innovative aspect of this result is that its non-cytotoxic effects on normal cells can be enhanced, and more effective action can be developed at lower doses against cancer cells when the crude extract is purified, thus taking advantage of the antimicrobial potential of these extracts, which can create a synergy of very interesting bioactivities for the pigment industry [89].

Other studies confirm the anticancer potential obtained from colored extracts and pigments of *Streptomyces*, as is the case of the red pigment corresponding to the compound undeciprodigiosin, which showed CA against HeLa cell lines [54]. This gives us an insight that many of the pigments isolated from *Streptomyces* may have an anti-cancer effect against tumor cells due to the intrinsic characteristics of the composition of the multiple secondary metabolic compounds that may participate in the mechanism of action of these extracts, fractions, or bioactive compounds [35].

This is confirmed by the hundreds of reference compounds that have been isolated from *Streptomyces*, such as doxorubicin, produced by *Streptomyces peucetius*, bleomycin produced by *Streptomyces verticillus*, and actinomycin D, produced by *Streptomyces parvulus* [35].

According to this behavior of extract 145, the importance of fractioning the extract is observed to achieve a better behavior of the secondary metabolite that is being synthesized and shows the bioactivity already present.

Most of the research reports ethyl acetate as the best solvent for separating or fractionating crude-colored extracts, obtained by biological fermentation and especially antimicrobial bioactive [171] .

In the literature, it is reported that *Streptomyces olivaceus LEP17* with pale yellow coloration said four spots with different Rf values: 0.64, 0.78, 0.93, and 1.00, showing bioactivity against *S.aureus*, *E.coli*, *P. aureginosa* and *Klebsiella sp* with inhibition halos between 11,15,13, and 12 mm [172]. GC-MS analyzed the bioactive fraction with Rf value of 0.93, and the component with the longest retention time and highest abundance was a cyclopentene [172]. Other investigations have reported new antimicrobial molecules from *Streptomyces* with structures of a cycloalkene, such as uncialamycin, cladoniamides A-G, and angucycline [173].

On the other hand, Subramanian et al. reported bioactivity of a yellow pigment, whose TLC characterization gave an Rf value of 0.45, showing bioactivity against *Pseudomonas sp.* Bioactive metabolites produced from *Streptomyces enissocaesilis SSASC10* against fish pathogens [174].

As indicated above in the antimicrobial activity results session with the fractionated extract. It could be observed in Figure 15 that the enriched extract 145 at a concentration of 0.3 mg/mL was able to inhibit 91% of the growth of *S. epidermidis*. This Gram-positive bacterium is of clinical relevance due to the implications or damage it can cause to human health [175].

In the literature, researchers report a purified active fraction of marine *Streptomyces* that achieved an 86.64% inhibition against methicillin-resistant *Staphylococcus aureus*, with a concentration of 8 μg/mL, surpassing the antibiotic action of the positive control vancomycin [176]. Although the attention of this fraction reported is much lower when compared with the extract of the study, it can be observed that in the qualitative assay (antibiogram), the potency of extract 145 at a concentration of 30 mg/mL maximizes its antimicrobial effect against *S. epidermidis*, which indicates that in this partial fractionation, the extract can be improved to a great extent, at the moment of performing a purification of the component or mixture of secondary metabolites that present the antimicrobial bioactivity, reaching to speculate that a pigment with antimicrobial activity close to 100% can be obtained, at doses equal to or lower than those established in the commercial antibiotics used as positive controls and those reported in other investigations.

However, a similar result was found in the growth curve assay reported by Erika Alves et al [177]. Their study consisted of the antimicrobial activity of a fractionated extract of *Streptomyces ansochromogenes* against *Pseudomonas aureginosa*, indicating inhibition

of 82.54% at a concentration of 0.25 mg/mL [177]. This means that the premise can be raised of working with a partially fractionated extract, which has long-term effects, which ends up being more feasible in the cosmetic industry since what is sought with cosmetic products is their long-term use, consumer loyalty, and therefore a future repurchase.

3.6 CONCLUSIONS

In summary, this study describes obtaining a colored extract produced by *Streptomyces* isolated from the Arauca River and belonging to the BioBank of the Universidad de La Sabana. The colored extract 145 has potential antimicrobial activity against *B. Sutilis*, *S.aureus*, *S. epidermidis*, and *E. faecium*; additionally, it has a free radical scavenging capacity of 10 mg/mL. On the other hand, it is shown an anticancer agent against HeLa cells, and its antimicrobial power is potentiated when fractionated in 100% ethyl acetate. It can be concluded that the fractionated extract of *Streptomyces* 145 meets the minimum requirements to be considered a possible cosmeceutical ingredient.

4 CHAPTER 4: BIOPRODUCTION, BIOACTIVITY, AND PARTIAL CHARACTERIZATION OF EXTRACELLULAR PIGMENT PRODUCED BY *STREPTOMYCES PARVULUS*

4.1 ABSTRACT

Actinomycetes are widely studied microorganisms, one of the most representative genera, *Streptomyces*, which produces 76% of the metabolites of most significant relevance and commercialization in the biotechnology industry. Among them, pigments stand out. These compounds benefit in the textile, food, cosmetic and pharmaceutical industries. Their importance lies in the fact that they strongly influence the consumer decision. However, most of them are not very favorable for human health. A solution to this can be found in obtaining pigments from *Streptomyces.* It's easy manipulation, scaling, and reports of pigments from this source, with excellent therapeutic properties, make it a promising alternative. The objective of this study was to improve the production conditions of the extracellular pigment generated by *Streptomyces parvulus*, evaluating the different variables (pH, incubation time, carbon and nitrogen concentration, agitation speed, and temperature) that could have a significant effect on the production of the secondary metabolite, using a factorial design (Plackett-Burman), to select the most effective terms. The independent variables that had the most significant impact were temperature, incubation time, and agitation speed. These factors were optimized using the Box-Behnken, giving optimal conditions: 30 ºC, 50 rpm, and seven days of incubation for maximum production of 465.33 μg/mL. On the other hand, refined chemistry processes were applied to partially identify the compounds or functional groups in the fractionated pigment (extracted with 100% ethyl acetate), evidencing the presence of C≡C, C≡N triple bonds, C−C, C−N, and O−H vibrations. The anti-tyrosinase activity gave a maximum inhibition of 10.94%, at a concentration of 300 μg/mL. The inflammatory activity reported a 36.89% inhibition against TNF-a interleukins; as for IL-10, there was a significant increase in its concentration concerning macrophages treated with lipopolysaccharide. These results highlight an adjusted model that allows scaling up the fermentation to an industrial level, providing optimal conditions that imply energy and raw material savings compared to other *Streptomyces* pigments, in addition to its biological bioactivities, to be a promising cosmeceutical ingredient.

Keywords: *Streptomyces*, Pigment, Antityrosinase, Optimization, Antiinflammatory, Anti-acne, Cosmeceutical

INTRODUCTION

Actinomycetes are the prokaryotes of most excellent scientific value since they have been the source of production in recent years of secondary metabolites with more applications in all types of industry, from pharmaceuticals, agriculture, and paint, cosmetics, textiles, and food, among others [118]. It is estimated that more than 23,000 secondary metabolites have been obtained from microorganisms, and approximately 10,000 of these bioactive compounds are generated by actinomycetes, 76% of which correspond to the *Streptomyces* genus [118]. For 50 years, research and programs have been developed around the isolation and discovery of new molecules from terrestrial actinomycetes, given the success of their biotechnological potential and the incredible biodiversity generated by these study areas [154]. Such has been the case of the Arauca and Guaviare rivers in Colombia, where study samples were obtained to consolidate the BioBank of actinomycetes that today has direction and development at the Universidad de la Sabana [154].

These riverine zones have been the subject of study in Colombia for more than ten years by educational institutions, given that they are places with many biodiversities to discover [154]. They have been little explored in terms of microbial biodiversity, which makes them attractive since there is an opportunity for Colombia to be a region that promotes biotechnological products with high impact in the world and that can be easily scaled, as is the case of the pigments that have been previously isolated from *Streptomyces*, and of which there are already reports of being promising sources of bioactivities [154].

Pigments are one of the biotechnological products of most significant interest to the scientific community. These compounds have taken on a crucial relevance, given their significant contribution to all industrial applications[178].

Since they not only make the product attractive but can also improve its qualitative properties, such as taste, quality, or durability of the mime [178]. In addition, it is estimated that by 2027, the demand for pigments in the world will have increased by 5% of what is currently sold, equivalent to $33.20 billion US dollars [178]. However, the collateral effect that organic or synthetic pigments have on the environment and human health must be considered [136]. Therefore, the importance of scaling up the production of pigments from natural sources that not only generate a product that is safe for human health but that is environmentally responsible and economically feasible becomes a requirement for the sector [179].

One of the main disadvantages of microbial pigment production is the low yield obtained in the standardization of the production process since a considerable amount of biomass and fermented culture is required to reach profitable crops on an industrial scale [15], [119], [120]. Therefore, variables such as pH, nitrogen sources, carbon, temperature, agitation, incubation period, and temperature can have a significant effect on the production of these pigments in crude form, which can be refined, fractionated, or purified, using the extraction methods that involve the lowest cost, but obtain the highest yield, so that it can be transformed into an affordable raw material in the market [15].

Following previous investigations of the crude extract 145, corresponding to a *Streptomyces* from the Arauca River, which reported promising extracellular coloration and cosmetic bioactivities, the purpose of this experimentation is to find the factors that affect the bioproduction of this pigment at laboratory scale, select the variables that maximize the yield of the production of the colored fraction and complement the cosmetic bioactivities already studied, with the evaluation of the anti-tyrosinase activity [180], [181], which is of great relevance in the cosmetic industry due to the aesthetic results that it can generate in a person with problems of melasma, sun spots, oxidative stress among other factors that affect the luminosity of the skin [181], and additionally, the anti-inflammatory activity that plays a fundamental role in the skin, since it is possible to develop products that can alleviate diseases such as dermatitis, eczema, decrease cortisol that can alter the tissues of elastin and collagen of the complexion [182], [183], or ultimately prevent clogging of pores that can cause acne [184], [185].

4.2 METHODOLOGY

4.2.1 Evaluation of the main factors that influence pigment production

Five carbon sources and five nitrogen sources were evaluated [45], [79], [186], [187], and the selection of the sources was based on previous studies on the improvement of *Streptomyces* cultures for pigment production [188]. The one with the most significant effect on pigment production was determined from these sources; two concentrations of each were chosen to be included as a study factor in the Plackett-Burman experimental design, which corresponds to a two-level factorial design whose purpose is to identify factors or independent variables that positively affect pigment production [15], [188]. The experimental design was developed in triplicate at a laboratory scale of 30 mL and using 30 mg/mL Biomass for each run. Supplementary files, Table 23 shows the six factors that were evaluated (pH, temperature, agitation speed, incubation time, carbon concentration, and nitrogen concentration), along with the number of experiments (12 experiments) that were performed with the experimental design mentioned above [15], [188]. Each factor was denoted by (1) and (-1), representing the levels at which the experiment was performed. From the Plackett-Burman (Table 23, supplementary files), the following first-order equation (Equation 1) was obtained [15].

Equation 4

$$Y = \beta_0 + \sum \beta_i \chi_i$$

Y represents the dependent variable (pigment production), β_0, is the model intercept, β_i is a linear coefficient, and χ_i are the independent factors or variables that were named in the design of the Plackett-Burman [15].

4.2.2 Box-Behnken design

The interactions between the variables with a more significant positive impact on the production of pigments were analyzed and improved using a box Behnken [15]. The experimental design consisted of 15 runs (scale of 30 mL and using 30 mg/mL). The factors will be classified in 3 levels (-1) as low, (0) as a medium, and (1) as high; all tests were performed in triplicate [15]. The results were adjusted with a linear regression of the response surface, applying the following equation of second-order polynomials (Equation No. 2) [15].

Equation 5

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ii} \chi^2{}_i + \sum \beta_{ij} \chi_i \chi_j$$

Y represents the dependent variable (pigment production), β_0, is the model intercept, β_i is linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction between coefficients and χ_i are the levels of independent variables [15].

Once the influence factors were determined, the second-order polynomial equation was validated, performing the experimentation again in the culture conditions obtained by the experimental design in a bioreactor of 1 Lt [189].

4.2.3 Characterization of the pigment

The pigment fraction was characterized using HPLC-L/MS, and FT-IR techniques, based on the following protocols: R. Parmar, N. El-Naggar, J. Muñoz, and others (respectively) [15], [190]. As mentioned in chapter 3, the crude extract was fractionated with 100% ethyl acetate to obtain the pigmented bioactive fraction, following the protocol described in section 3.3.6.

4.2.4 Verification of the pigment *Streptomyces*

- **16S rRNA gene sequencing**

The sequencing of the extracted DNA was done with the 16S rRNA gene, following the protocol described in the GF-1 Bacterial DNA extraction kit (Vivantis Technologies, Selangor, Malaysia). The 16s rRNA gene was amplified by polymerase chain reaction (PCR) based on the extracted DNA using oligonucleotides 27F and 1492R, as described by Natalia Pastrana [153].

4.2.5 Tyrosinase inhibitory activity

This assay followed the protocol guidelines Cha et al., with some modifications. Tyrosinase inhibitory activity was determined using L-Dopa and mushroom tyrosinase as substrate [191]. In a 96-well plate, 100 μL of 200 Units/mL tyrosinase in a 25 mM buffer solution at pH 6.8 was added to each of the tested concentrations of the bioactive fraction 3, 30, 300, and 1000 μg/mL (50 μL volume) [191]. Then, 100 μL of the substrate was added at a concentration of 2.5 mM [191]. Finally, the absorbance was read in 10 cycles of 70 seconds at 490 nm. Kojic acid was used as a positive control at the following concentrations: (50, 100, 250, and 50 μg/mL) and 3% DMSO as a negative control; each engagement was mounted in triplicate [191]. The quantification was determined with the following equation [191].

Equation 6

$$\%\,Inhibition = \{[(A-B)-(C-D)]/(A-B)\}$$

A: Absorbance of the enzyme, substrate, and DMSO solution.
B: Absorbance of the substrate and DMSO solution.
C: Absorbance of the enzyme, substrate, and extract solution
D: Absorbance of the substrate and extract solution.

4.2.6 Anti-inflammatory activity

Anti-inflammatory activity was carried out using the following methodology described by Ruilin Zhang and his collaborators [183]. The TPH-1 cell line (ATCC® TIB-202™) was placed in 24-well plates at $1x10^6$ cells per well. They were maintained in culture in RPMI medium, supplemented with 10% fetal bovine serum (SFB) (Invitrogen) for 24 h. THP-1 monocytes were differentiated to macrophages by incubation for 48 h with 13-acetate 12-phorbol myristate (PMA) 150 nM, followed by 24 h incubation in RPMI medium [183].

Macrophages attached to the bottom of the wells were washed three times with 600 µL of PBS (0.1 M phosphate buffer, pH 6.4), incubated with SFB-free RPMI medium, and lipopolysaccharide (LPS) 50 ng/mL for four h to stimulate the inflammatory response [183], [192]–[194]. LPS-stimulated THP-1 macrophages were incubated for 24 h of treatment with RPMI medium and extract or fraction at a concentration of 300 µg/mL. At the end of the time, supernatants of monocytic THP-1 cells, THP-1 cells differentiated to macrophages, THP-1 macrophages with LPS, and THP-1 macrophages with LPS and extract or fraction were collected. Ibuprofen (Sigma Chemical Company (St. Louis, MO)) at 50 µg/mL for 24 h after inflammation with LPS was used as a positive control for anti-inflammation [183], [192], [194].

TNF-α and IL-10 levels in the supernatants were evaluated with the enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. The plates were read with a microplate spectrophotometer at an absorbance of 450 nm [183].
The concentration was calculated using standard curves. Each determination was performed in triplicate [183].

4.2.7 The antiacne activity of the fractionated extract

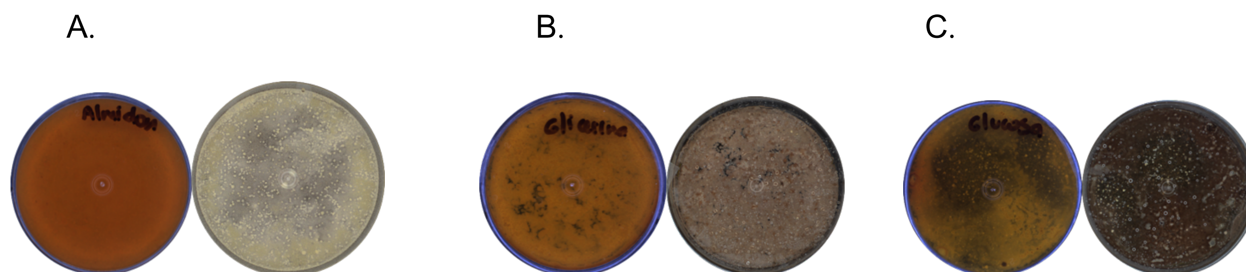
Based on the antimicrobial activity described previously in chapter 3 of this research, the anti-acne activity was again evaluated qualitatively against *S. epidermidis*, using the disk diffusion technique, considering a single concentration (10 mg/mL), following the MacFarland scale at 0.5% to establish the concentration of the bacteria, and taking Vancomycin as a positive control. As a negative control, the solvent was used to dissolve the fractionated extract (ethanol 30: Water 70) [184], [185]

4.3 RESULTS

4.3.1 Evaluation of the main factors that influence pigment production

4.3.1.1 Carbon source selection

Five carbon sources were evaluated: glucose, starch, lactose, glycerin, and sucrose, under conditions of 30 ºC and seven days of solid fermentation. In Figure 16, it can be observed that the carbon source with the most significant influence on the production of strain 145, which concentrates more yellow-orange color in 7 days, corresponds to soluble starch during the incubation follow-up. This source, on the third day, had more significant culture formation. After five days, the colonies were wholly formed, matured, and fully pigmented. The opposite was the case with sucrose, which presented a low, irregular, and prolonged growth rate. On the other hand, good growth was observed in glucose, lactose, and glycerin; however, only until day 5 was a homogeneous, almost mature layer followed, and at seven days, it was possible to see complete growth of the strain. ISP2 medium without starter was used as a negative control, which showed no change.



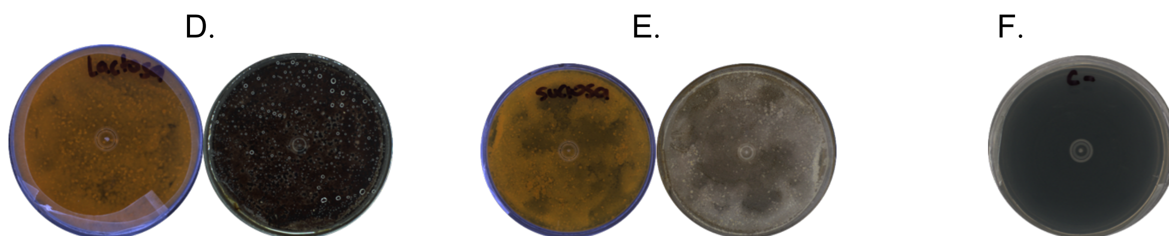


Figure 16 Effect of carbon sources on the growth of Strain 145. A) Soluble starch, B) Glycerin, C) Glucose, D) Lactose, E) Sucrose, and F) Negative control (ISP2, without inoculum). Each carbon source evaluated was carried out in triplicate. The result is shown in two images for each source referring to the front and back of the plate.

4.3.1.2 Nitrogen source selection

Five sources of nitrogen were evaluated: ammonium sulfate, potassium nitrate, ammonium chloride, yeast-malt, and casein, under the same culture conditions to which the carbon source was exposed. During the fermentation process, it was observed that the standard references used in preparing the ISP2 medium are the most adequate for producing the extracellular pigment produced by strain 145. As shown in Figure 17, no growth was observed in the potassium nitrate, ammonium sulfate, and ammonium chloride agar. An increase was observed in casein and yeast-malt; nevertheless, due to growth efficiency, there was a faster proliferation of malt and yeast in a short time; on the third day, growth was already more accelerated than in casein; nevertheless, the difference in growth was of 1 day. However, considering the cost of the raw materials, the cost benefits of yeast and malt concerning casein were highlighted.

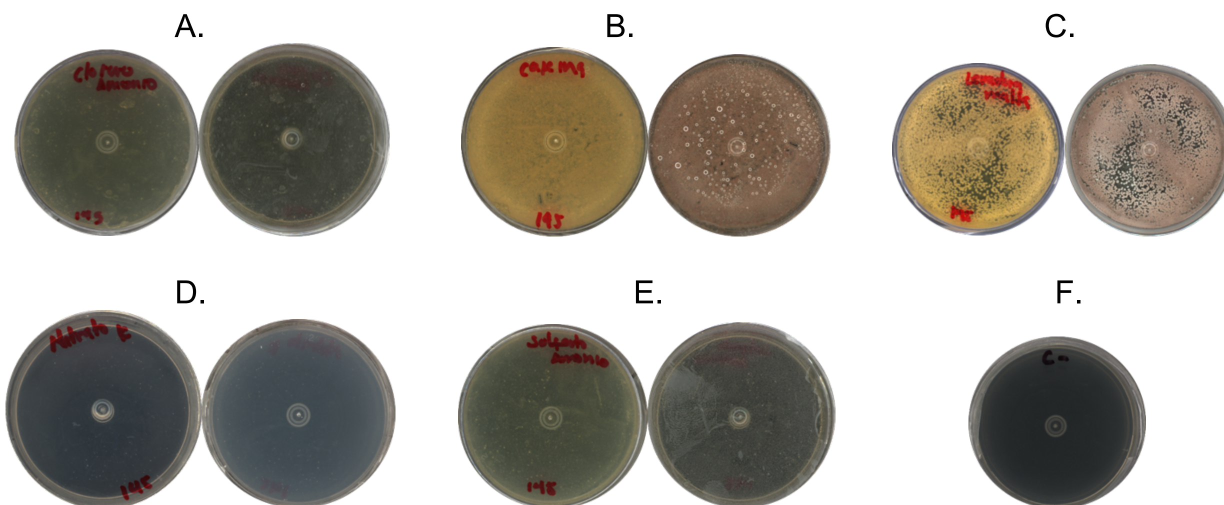


Figure 17 Effect of nitrogen sources on the growth of strain 145. A) Ammonium chloride, B) Casein, C) Yeast/malt, D) Potassium nitrate, E) Ammonium sulfate, and F) Negative control (ISP2, no inoculum). Each nitrogen source evaluated was carried out in triplicate. The result is shown in two images for each source referring to the front and back of the plate.

4.3.1.3 Plackett-Burman experimental design

The selected carbon and nitrogen sources were used to carry out the design of experiments, which determined the factors that significantly affected pigment production. The trial was carried out in 12 runs, with different factors such as temperature, pH, carbon source concentration, nitrogen source concentration, agitation, and fermentation time. Supplementary file (Table 23) shows the scheme of how the experimental design was set up. Table 16 shows the results obtained in the response variable under the most significant effects.

Table 16 Plackett-Burman experimental design of 12 trials for the evaluation of 6 independent variables with coded values along with pigment production. T (temperature); pH; It (Incubation time); rpm (stirring speed); NC (nitrogen concentration); CC (carbon concentration).

Run	level	T	pH	It	NC (%)	CC (%)	rpm	Concentration ug/mL
1	1	35	6	7	0.1	0.1	100	85.33
2	-1	25	8	7	0.1	0.2	100	13.33
3	1	35	8	3	0.2	0.1	100	43.40
4	1	35	6	7	0.1	0.2	200	7.40
5	-1	25	8	7	0.2	0.2	100	62.00
6	1	35	6	7	0.2	0.1	200	36.07
7	1	35	8	3	0.1	0.2	200	12.00
8	1	35	6	3	0.2	0.2	100	6.73
9	-1	25	6	3	0.2	0.2	200	60.07
10	-1	25	6	7	0.2	0.1	200	70.73
11	-1	25	8	3	0.1	0.1	200	78.73
12	-1	25	6	3	0.1	0.1	100	0.07

The Plackett-Burman results showed a significant variation in pigment production, as shown in Table 16. The run with the lowest concentration was obtained at operating conditions such as 25 °C, pH 6, three days of incubation, equal attention in both sources (0.1%), and 100 rpm, resulting in 0.07 μg/mL. On the other hand, the strong influence of factors such as temperature, pH, rpm, and incubation time can be seen, as indicated by the result of run 1, which gives the highest pigment concentration, 85.33 μg/mL. The multiple regression mathematical model relating the factors or independent variables to pigment production reveals that the nitrogen source concentration factor is a non-significant variable with a null effect since the p values are more significant than 0.05.

This can be interpreted more explicitly with the Pareto diagram, where the influence of the factors: agitation, pH, temperature, and incubation time are seen to have a greater significant effect. On the other hand, the results of the multiple regression of the

mathematical model and its respective coefficient of determination adjusted R, and predicted R are presented in Table 17.

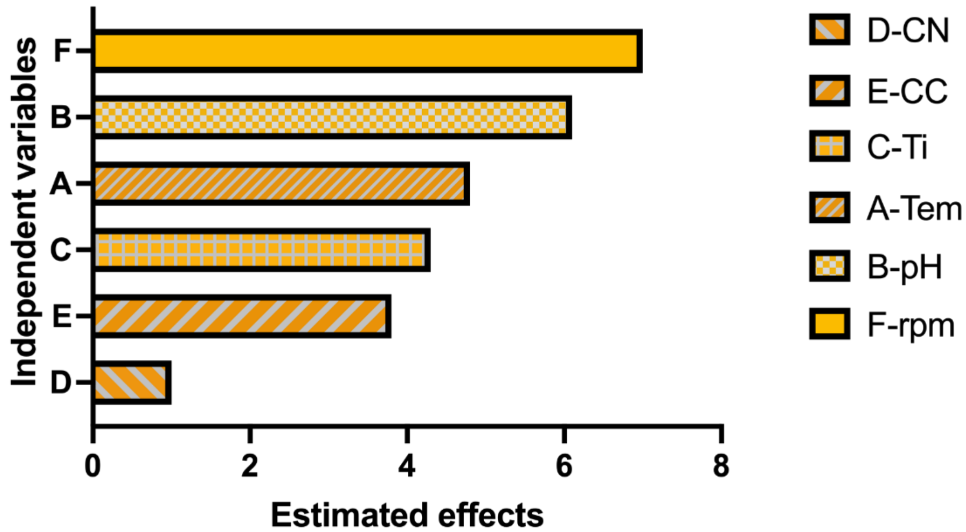


Figure 18 ***Pareto plot: Estimated effects on the independent variables****, A (Temperature), B (pH), C (Incubation time), E (Carbon concentration), D (Nitrogen concentration), F (agitation).*

Table 17 Factorial regression of the response variable: Pigment concentration (µg/mL) vs. Variables: Temperature, pH, N concentration, C concentration, agitation, and incubation time.

ANOVA					
Source	DF	Adjus SC	Adjus MC	F value	p value
Model	6	10314.6	1719.1	24.12	0.002
Lineal	6	10314.6	1719.1	24.12	0.002
T	1	1587.0	1587.00	22.27	0.005
pH	1	2760.3	2760.33	38.74	0.002
IT	1	1382.5	1382.45	19.4	0.007
NC	1	62.20	62.2	0.87	0.393
CC	1	1077.7	1077.69	15.12	0.012
rpm	1	3444.92	3444.92	48.34	0.001
Error	4	71.26	71.26		
Total	11				
	S		R-cuad	R-cuad. (Adjust)	R-cuad. (Pred)

8.44148	96.66%	92.65%	80.77%

By excluding the terms that are not significant in the model, the following first-order polynomial equation is obtained, which represents the production of yellow-orange extracellular pigment from *Streptomyces* sp. strain 145 as a function of the following: independent variables:

$$\mathbf{Y = 39.66 + 11.50Tem - 15.17pH + 10.73Tin + 9.48CC + 16.94rpm}$$

Where Y is the response variable (concentration of pigment produced, expressed in ug/mL), T (temperature), pH, It (Incubation time), CC (carbon source concentration), and rpm (agitation).

From this first-order polynomial equation, we optimized the model using a response surface design (Box-Behnken) and selected the factors with the most significant importance or positive effect on the response variable. As can be seen in the previous statistical analyses and the model provided, the most relevant factors were temperature, agitation time, and agitation speed; with these three factors, 15 experimental runs were carried out in triplicate, as can be seen in Table 18, with their respective coding and pigment production value.

Table 18 Box-Behnken response surface design representing the extracellular pigment production of Streptomyces sp. *strain 145 under the influence of Temperature, rpm, IT, Concentration of the colored crude extract.*

Run	Temperature	rpm	It	Concentration µg/mL
1	30	100	9	445.5
2	30	150	7	142.7
3	40	50	7	42.7
4	40	100	5	76.4
5	35	100	7	210.4
6	30	50	7	440.1
7	35	50	5	247.4
8	40	100	9	205.4
9	35	100	7	304.1
10	35	150	9	8.7
11	40	150	7	15.4

12	35	50	9	284.4
13	35	150	5	7.4
14	35	100	7	392.4
15	30	100	5	55.4

With the results reported in Table 18, a relationship between dependent and independent factors is made to determine the maximum production of yellow-orange pigment corresponding to the optimum values of temperature, agitation, and incubation time, obtaining the following second-order mathematical model:

$$Y = 227.28 - 201.51A - 71.30B + 115.35AB - 150.88B^2$$

Where Y corresponds to the concentration of extracellular pigment at the optimum temperature and agitation speed conditions, the incubation time ceases to be relevant in the mathematical model when evaluating the interaction of main effects; therefore, in the regression, there is no presence of this variable.

The results of the model adjustment are presented in Table 19, where the sum of squares can be observed, indicating the type of polynomial that best fits the system of variables evaluated, the value of F, and the residual information.

*Table 19 Regression statistics, analysis of variance, for Box-Behnken results used for optimizing pigment production by Streptomyces sp. strain 145. *Significant values, df: Degree of freedom, F: Fishers's function, P: Level of significance, C.V: Coefficient of variation.*

Source	Sum of Square	df	Mean Square	F-value	p-value
Model	3.117E+05	1	77920.09	22.66	<0.0001*
A-Temperature	1.367E+05	1	1.367E+05	39.76	<0.0001*
B-rpm	27109.21	1	27109.21	7.88	0.0185*
AB	27888.84	1	27888.84	8.11	0.0173*
B2	78613.75	1	78613.75	22.87	0.0007*
Residual	34381.57	10	3438.16		
Lack of Fit	17814.71	8	2226.84	0.2688	0.9279
Pure Error	16566.86	2	8283.43		
Cor Total	3.461E+05	14			
St Dev.	58.64	**R-squared**	0.9006		
Mean	191.81	**Adj R-squared**	0.8609		
C.V %	30.57	**Pred R-squared**	0.7914		

PRESS	72193.05	Adeq precision	14.4596

Three-dimensional plot

The response surface was plotted using a significant statistical model, which helped to understand more broadly the main effects' interactions and their respective optimum conditions to maximize the production of extracellular pigment of *Streptomyces* 145. As a result, Figure 19 shows the behavior of pigment production concerning temperature and agitation.

Maximum concentration production occurred near the edge below the surface at the top-left. Another pronounced curvature is at the lower-right corner. Therefore, the top points at seven days of incubation correspond to 30 ºC and 50 rpm, giving a pigment production of 465.55 μg/mL. As for the lower pigment point, it occurs at the conditions of 37 ºC, 113 rpm, and a concentration of 189.09 μg/mL. In the contour map (Figure 19-B), the design points and their respective curvatures in the maximum, central and minimum zones can be observed in more detail.

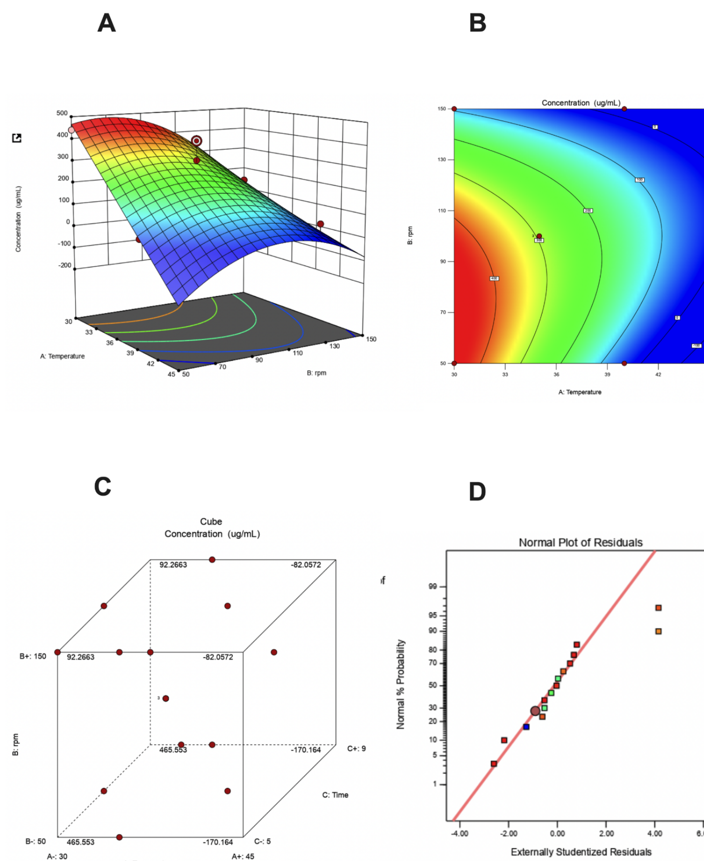


*Figure 19: **Results of the optimization of the extracellular pigment production of Streptomyces sp. strain 145**. A) Three-dimensional response surface plot. B) contour map for pigment production of Streptomyces sp. strain 145 showing the interactive effects of temperature, stirring speed, and fixing the incubation time variable. C), Pigment production concentration cube at the different design points. D) Normal plot of the residuals, indicating the quality of the optimized model.*

The model was validated by culturing *Streptomyces* sp. Strain 145 in a 1L bioreactor at the optimal conditions provided by the regression. The model error was 1.72%, and the concentration obtained at the laboratory level was 457 μg/mL, very close to the value obtained from the simulation, which corresponds to 465.33 μg/mL.

Characterization of the pigment

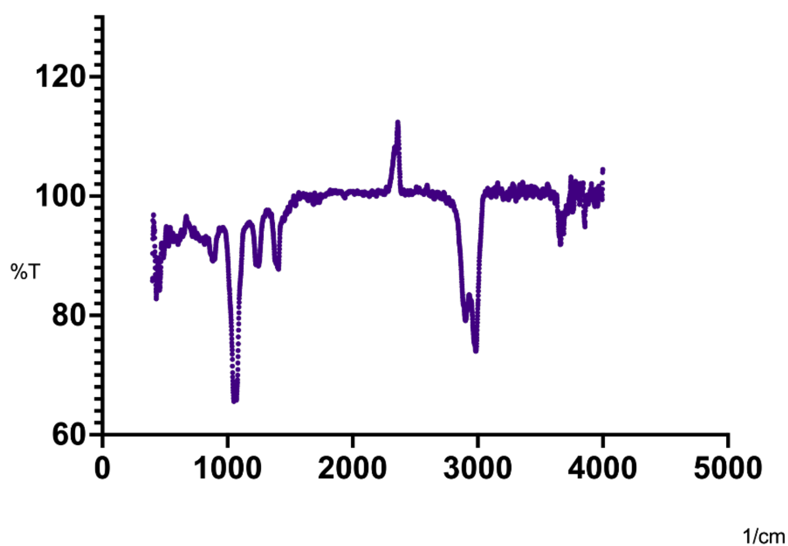


Figure 20 FTIR of the pigmented fraction of Streptomyces sp. *strain 145, obtained from laboratory-scale validation at appropriate conditions according to optimization. This fraction was extracted with 100% ethyl acetate and solubilized in water:ethanol solvent (70/30 v/v).*

Consequently, the FTIR spectrum of the fractionated pigment 145, exhibited the following absorption peaks at 3001, 1075, 1441, 2903, 2381, and 3682. cm^{-1}, as the highest and most representative of it.

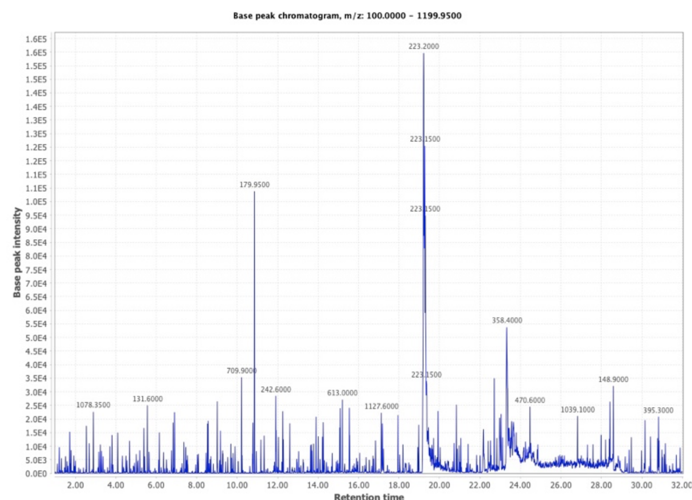
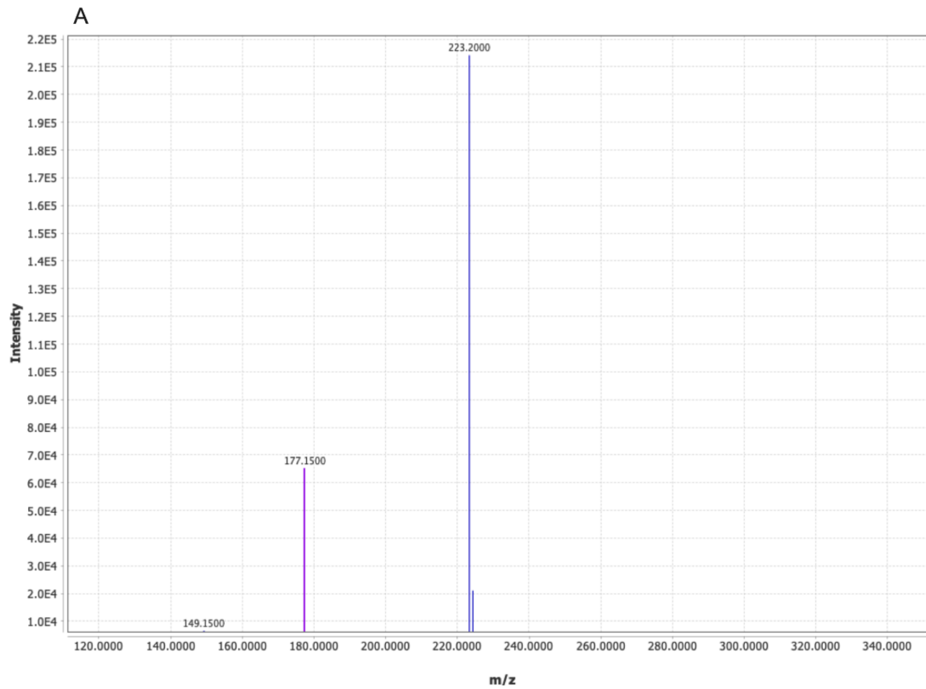


Figure 21 HPLC-L/MS spectrum of the pigmented fraction of Streptomyces sp. *strain 145, obtained from laboratory-scale validation at appropriate conditions according to optimization. This fraction was extracted with 100% ethyl acetate and solubilized in water:ethanol solvent (70/30 v/v).*

Figure 21 shows the chromatogram of the metabolite or metabolites of interest of fraction 145. Several peaks are observed. However, those with the longest retention time are highlighted, being reduced to only three peaks with the construction of the chromatogram and its respective deconvolution, as can be seen in Figure 22 with the help of the MZmine program.



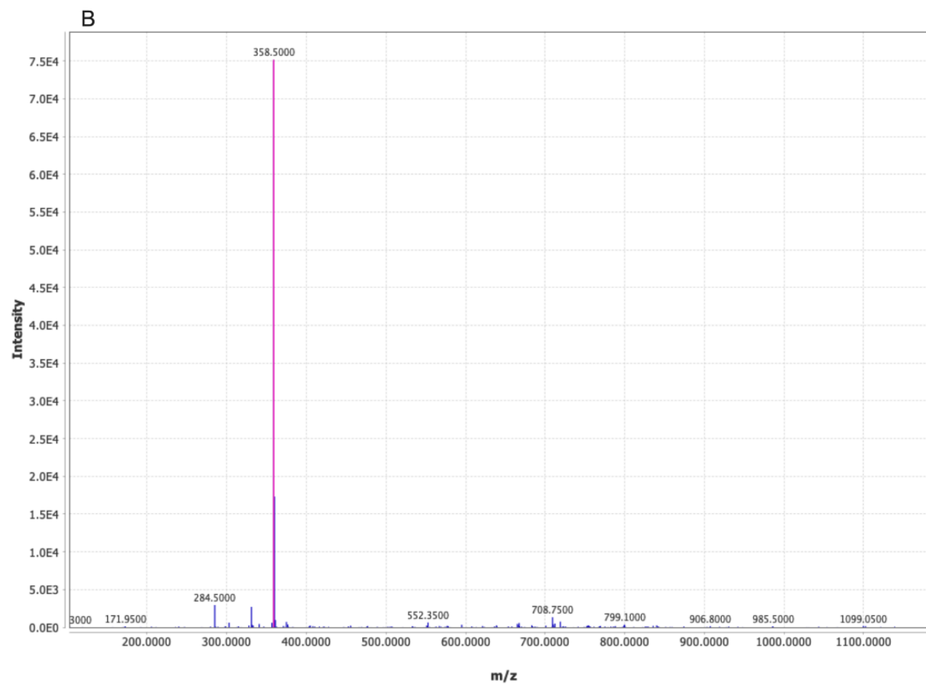


Figure 22: ***Mass spectral deconvolution of the pigmented fraction of Streptomyces sp 145 strain.*** *base peak of the highest intensity signals in the deconvoluted chromatogram of fraction 145.*

4.3.2 Verification of the pigment *Streptomyces*

Strain 145 was identified using the 16S rRNA gene sequencing technique. The results showed a 98.7% similarity value, indicating that the study microorganism corresponded to *Streptomyces parvulus*.

4.3.3 Tyrosinase inhibitory activity

The effects of the antityrosinase activity of the *Streptomyces parvulus* strain extract can be seen in Figure 23, clearly reflecting an inhibition that increases with time and concentration. However, kojic acid shows better inhibition throughout the spectrum of concentrations evaluated.

The results of tyrosinase inhibition vs. concentration of the colored fraction and the positive control are summarized in Figure 23.

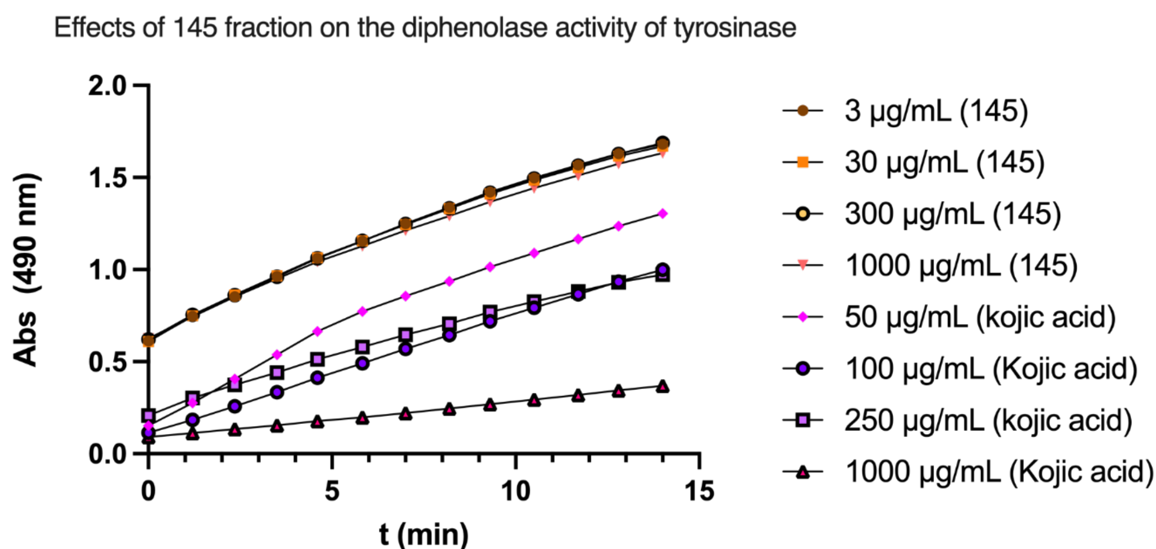


Figure 23 ***Effects of the pigmented fraction, extracted with 100% ethyl acetate, obtained from Streptomyces*** *sp.* ***strain 145 on tyrosinase diphenolase activity.*** *The enzyme activity was tested in the presence of L-DOPA as substrate Results are presented as means ± SD of four experiments. *p < 0.05, **p < 0.01, vs. untreated controls, Student's t-test. Ka, kojic acid; 145, the fraction of extracellular pigment produced by Streptomyces parvulus strain.*

In Figure 24, it can be observed that there is a significant difference between the evaluated concentrations of the fractionated pigment, with a significance level of $p < 0.05$. The colored fraction reported its maximum inhibition at 10.94%, at the maximum concentration evaluated, this being the concentration with significance concerning 3 and 30 μg/mL, contrary to the 30 μg/mL concentration, which did not show a significant difference with the 300 μg/mL concentration.

As for the control corresponding to kojic acid, the notorious change in the inhibition of tyrosine diphenolase activity is observed since the higher the concentration, the higher the inhibition power.

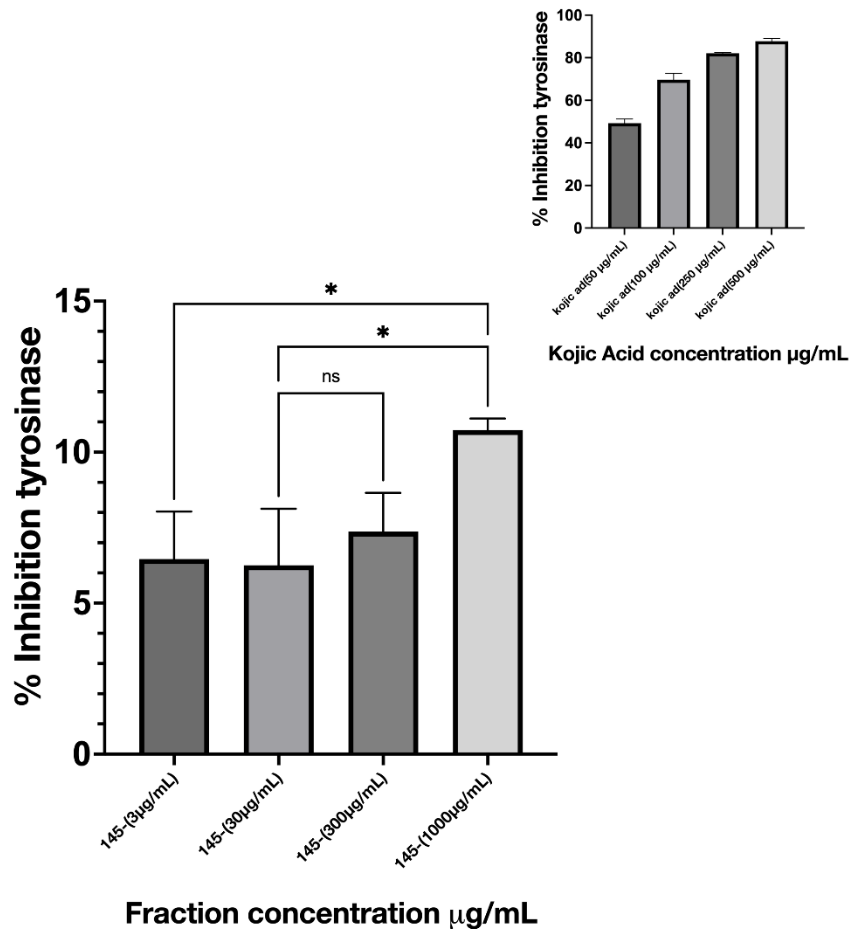


Figure 24 Different concentrations of the pigmented fraction of Streptomyces parvulus (145 strain) and the respective control (kojic acid) were incubated with the fungal tyrosinase. After incubation, the amount of dopachrome produced was determined spectrophotometrically at 490 nm. Each treatment was carried out in triplicate (p<0.05).

4.3.4 Anti-inflammatory activity

Cells treated with *Streptomyces parvulus* extract and fraction showed a significant difference ($p < 0.05$) in the concentration of proinflammatory cytokines released compared to macrophages treated with lipopolysaccharide. Additionally, a significant difference ($p < 0.05$) was found between the treatments at the different concentrations evaluated. Figure 25 shows the behavior of each treatment compared to the control.

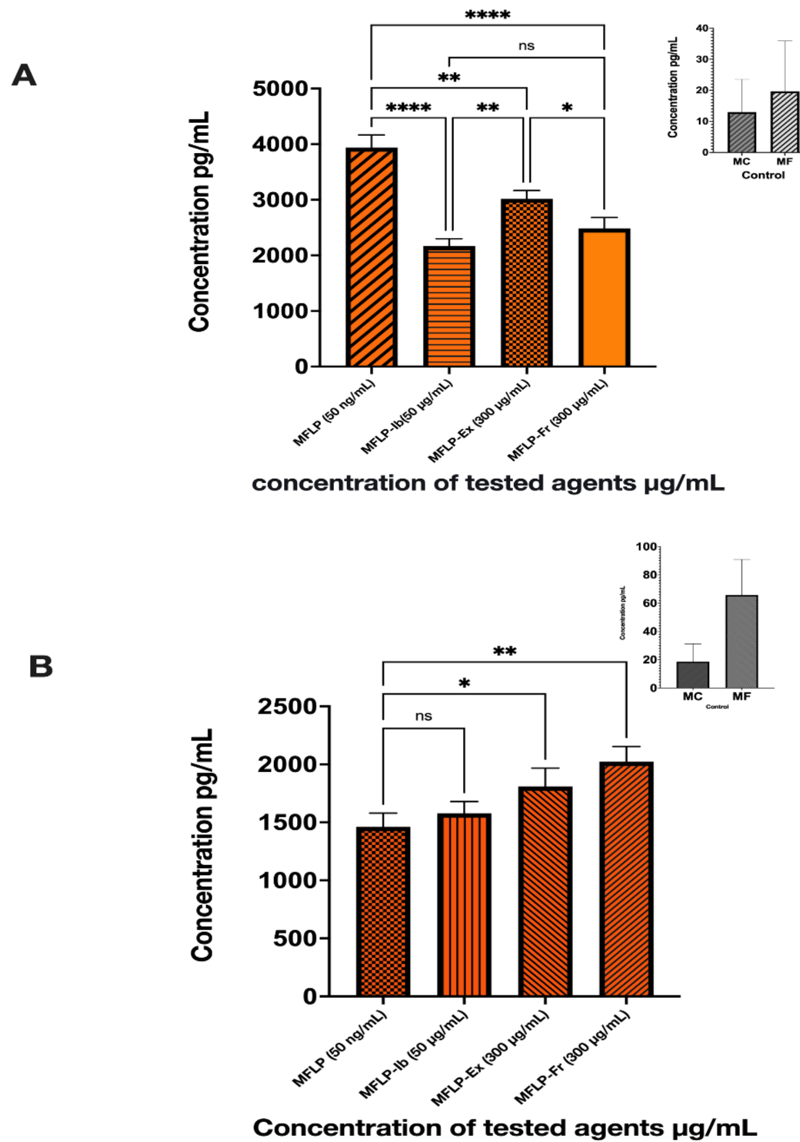


Figure 25 Effect of aqueous/ethanolic colored extract and pigment fraction of Streptomyces sp. Strain 145 (Streptomyces parvulus) on cytokine production. A. TNF-α; B. IL-10. "" indicates significant statistical differences (p < 0.05) between treatments and control, determined by Dunnett's statistic. Paired lines indicate statistical differences between concentrations of the same extract and treatments (p < 0.05) with Tukey's multiple range test.*

4.3.5 Antiacne activity

The results of the antimicrobial activity by disk diffusion technique can be seen in Figure 26, where a positive inhibition of *S. epidermidis* bacteria is shown when exposed to the fractionated extract of *Streptomyces parvulus* at a concentration of 10 mg/mL.

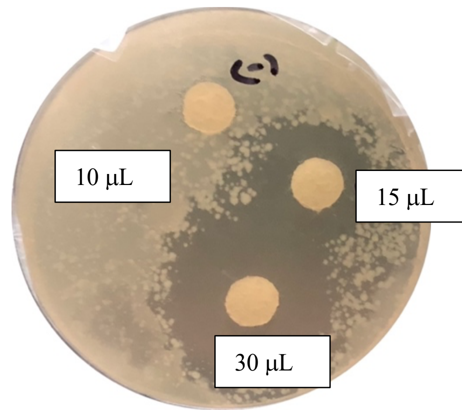


Figure 26 The evaluated concentration of Streptomyces parvulus fractionated pigment against S. epidermidis, 70% water/ 30% ethanol (negative control); volumes tested: 15 uL and 30 uL at 10 mg/mL. Inhibition halo (90.2 mm-30 µL), (54.6 mm).

DISCUSSION

Streptomyces has three stages of growth; the first stage corresponds to the formation of spores [195]. In the initial spore stage, the construction of germ tubes is observed, which rapidly extend in the form of branches, giving the appearance of a dense network of hyphae, which is known as vegetative mycelium and is the initiator of stage 2, where the most significant consumption of nutrients occurs, and aerial hyphae are formed [195]. In stage 3, these aerial hyphae become large spore chains [195].

This growth can be altered by modifying the culture media used for bacterial growth [196]. One of the simplest ways to observe if there is an effect on bacterial growth is to change the source of carbon and nitrogen [15], [196]. As shown in Figures 16 and 17. Figure 16 represents which carbon source contributes positively to the more accelerated and concentrated growth of the microorganism and its respective extracellular pigment. Starch was found to be the carbon source with the most significant influence on the production of the yellow extracellular pigment. Carbon sources can often be assimilated very quickly by microorganisms or sometimes slow down their metabolism, having an essential effect on the biosynthesis of secondary metabolites [196].

Soluble starch, a homopolysaccharide, has the characteristic of hydrolyzing more slowly than glucose. Its absorption is prolonged compared to glucose, which decreases catalytic repression, thus increasing pigment production [120]. On the other hand, the nitrogen sources, as shown in Figure 17, do not present significant changes when compared to the yeast/malt extract, which is the original composition of the medium. This makes it even more attractive because yeast and malt are the most economical sources for bacteria to have an efficient and quality growth due to their fast absorption [120]. Yong Zhou and coworkers report a similar result with a glycoprotein produced by *Streptomyces kanasenisi* ZX0, whose yield is enhanced in the presence of soluble starch and yeast extract [120].

By the influence that the nutrients of the medium have on the production of the metabolites of interest, other variables affect the biosynthesis of this, as we can observe in the session of the evaluation of the main factors that affect the production of pigment [15]. The experimental design of Plackett-Burman exposes the variables that contribute the most significant effect to the model, being the agitation speed, the term of greater significance in the metabolite of interest, reporting a p-value of 0.001; on the other hand, Table 17. summarizes the ANOVA, of the factorial regression, indicating that 0.9666, affirms that 96.66% of the variability in the response, is attributed to the factors or independent variables studied [15]. Additionally, the R2 is very high. Therefore, the premise that the model has significant relevance is maintained. This is corroborated by the F value of the model, 24.12, with a p-value of 0.002. Therefore, the model obtained with the Plackett-Burman, exposes temperature, incubation time, and agitation as the factors with the most significant positive influence, which will give the optimum conditions for maximum pigment production that can be scaled at the laboratory level.

The mathematical model generated by the Box-Behnken experimental design yields a second-order polynomial equation, where it is evident that there is a strong influence on the interaction effects of the independent variables (temperature: agitation time). The Fisher statistic in Table 18 and the p-value imply that the model is significant. The linear coefficients, interaction coefficients, and quadratic effect of agitation are important terms. Therefore, there is a significant correlation between the two variables that turned out to have an optimal value in the design for pigment production. However, the variables temperature and agitation showed a VIF greater than 1, indicating high multicollinearity. As for the model fit, the model presented an excellent coefficient of determination with an R2 of 0.9006, indicating a reliable model for extracellular pigment production by *Streptomyces parvulus* in the present study. Additionally, Pred-R2 and Adj-R2 have a difference of 0.07; this reaffirms a good fit between the observed and predicted values. On the other hand, the model gives a good (Adeq accuracy) of the noise signal, being 14.459, higher than the ratio of 4, which is the most desired [15].

The optimal conditions determined by the model are the lowest in terms of cost. Therefore, fermentation is possibly feasible to scale up since it will not require a robust agitation system or high energy costs. Suppose a quick economic analysis is made of what it costs at the laboratory level to model this fermentation of 7 days of incubation, 30 °C, and 50 rpm. In that case, the following scenario is found: the cost of the culture medium for one liter of fermentation would be COP 5,985 (Colombian peso), the energy cost for seven days is COP 130,198, as predicted by the model, and the maximum production that would be reached is 465. 33 μg/mL, the production cost per gram of pigment would be $135.71/μg; if it is proposed to work with a crude extract, it would be an economically affordable compound compared to pigments such as melanin, which is expensive to produce [15].

Regarding the molecular identification of the evaluated fraction, asymmetric and symmetric vibrations are observed in the 3001 and, as 2903 cm^{-1} bands seen in Figure 20. On the other hand, C−C, C−N, and C−O stretches are present in the 1075-1441 cm-1 band. Usually, it is not very frequent to see signals in the 2000-2500 region; they are

generally very weak; however, at 2381 cm-1, a sharp peak is formed, which indicates the presence of triple bonds, such as C≡C, C≡O, and C≡N. Complementing what the FTIR shows, with the LC-MS spectrum, three peaks of greater intensity are observed, making their respective analysis in libraries that report compounds produced by *Streptomyces* (StreptomeD) , desferrioxamine I1b is put as a possible candidate since the fragmentation pattern reported for this compound, shows m/z of 177.1500 and 223.200 [197]. Additionally, it has biological properties very similar to those found in this study [198]. Another candidate for the peak observed at 358,500 corresponds to compound BE 18591 [199]; reviewing its structure and the FTIR bands, the presence of this compound in the pigmented fraction can be presumed. In addition, a study by Keiji Tanigaki and collaborators found the anti-inflammatory activity of this compound, which may explain the inflammatory activity of the pigmented fraction 145, which will be discussed later [199].

The biological activities evaluated with the pigmented fraction of *Streptomyces parvulus* showed results that reaffirm the therapeutic potential of this metabolite or mixtures of secondary metabolites in the cosmetic industry. Figure 23 shows the diphenolase activity of the fractionated pigment, which, although, indeed, it does not exceed the positive control (kojic acid), highlights its inhibition power in the first 5 min, at the highest concentration of 300 μg/mL, which can be very attractive in the case of adding this fraction to a cream that meets the cosmeceutical characteristics or for dermatological use, working in the depigmentation of age spots, sun or as a promoter of illumination, with a progressive, long-term effect. However, it is essential to mention that a competitive advantage that the evaluated fraction may have over kojic acid is the stability shown by the latter during the test; its tyrosinase inhibition did not reach more than 11% but remained constant throughout the reaction; this is interesting because there is a possibility that the *Streptomyces parvulus* pigment is less sensitive to light, as opposed to kojic acid, which quickly loses its depigmenting potential due to its sensitivity to light and air, reducing it to a very unstable molecule. In addition, there is a concern about its possible adverse effects on human health; some research has indicated that it may be potentially cytotoxic and irritating to the skin [200].

Regarding the anti-inflammatory activity, the *Streptomyces parvulus* pigment showed a percentage of 36.89% inhibition of pro-inflammatory interleukins TNF-α against macrophages stimulated with lipopolysaccharide, a result very close to ibuprofen (44.99%), as shown in Figure 25, the reference compound used, due to its anti-inflammatory properties, previously reported [201]. An interesting aspect that stands out in this result is that there is no significant difference between the pigmented fraction and ibuprofen, so it could be speculated that the extracellular pigment contains compounds that generate mechanisms of disinflammation against this type of cytokines. Another similar result is reported in the research conducted by Ravinder Polapally and collaborators; whose evaluated compound was Melania, extracted from *Streptomyces puniceus RHPR9*, at a dose of 500 μg/mL, showed a 21.37% anti-inflammatory activity through the inhibition of lysis of the cell membrane of red blood cells [89]. The result of fraction 145 is more attractive in contrast to that provided by melanin since a lower concentration was used, and better inhibition of interleukins was presented. In addition,

this test specifies the type of cytokine that was reduced; on the other hand, it is essential to note that not only the fraction showed anti-inflammatory activity, but the crude extract also showed a potential anti-inflammatory effect of 23.34%, very close to that reported by melanin [89]. On the other hand, the pigmented fraction, and the extract, as shown in Figure 10B, showed a significant increase concerning lipopolysaccharide-treated macrophages in IL-10 encoding; this reflects a positive result that is worth exploring further since IL-10 is known to be a potent anti-inflammatory cytokine that can reduce inflammation or regulate the production of proinflammatory interleukins [202].

Finally, the anti-acne activity was corroborated using the disc diffusion technique, evaluating a lower concentration of the fractionated pigment to determine whether, once the optimal conditions were standardized, bioactivity was not lost. Figure 26 shows that the extract presents antimicrobial activity against *S. epidermis*. This result reaffirms obtaining a partially fractionated, colored extract with antimicrobial potential, which, together with the other evaluated activities, complement each other to be a possible candidate for use in the cosmetic industry.

4.4 CONCLUSIONS

The results of the present study reveal that the fractionated pigment of *Streptomyces parvulus* presents optimal conditions of 30 ºC and 50 rpm at seven days of incubation, these being the variables with the most significant impact on optimization. Additionally, the model obtained by the Box-Behnken experimental design validated in the laboratory gave an error of 1.72%, which is very positive in a biological model. On the other hand, the physical activities indicate the depigmenting, anti-inflammatory, and anti-acne potential of the fractionated pigment. Finally, the presence of compounds such as desferrioxamine and BE 18591 is speculated to be the possible generators of the biological activities evaluated.

5 CHAPTER 5 GENERAL CONCLUSIONS

Streptomyces strains isolated from riverine soils of the Arauca and Guaviare rivers were selected for liquid fermentation at 35°C, 150 rpm, and seven days of incubation to determine the presence of extracellular pigment. Of the 20 strains evaluated, only 3 pigmented the medium, corresponding to the following coding: 145, 290, and 627. These three strains were subjected to preliminary bioactivity assays, which evaluated antimicrobial, antioxidant, and cytotoxic activity. Strains 145 and 290 showed antimicrobial potential against pathogens such as *B. subtilis*, *S. aureus*, *S. epidermidis*, and *E. faecium*. Strain 627 did not show any antimicrobial activity. On the other hand, strain 627 had a better percentage of free radical uptake, with 53.70% at 10 mg/mL, compared to strains 145 and 290, with 22.06% and 5.27%, respectively. Regarding cytotoxic activity, strain 145 presented an attractive antineoplastic potential against HeLa cells, with an IC_{50} of 37.34 μg/mL. Also, there was an excellent cell viability behavior against human fibroblast cells by strain 627, presenting 0% cell death. Based on the preliminary bioactivity results, it was decided to work with the crude color extract of strain 145, as it had a better antimicrobial, cytotoxic and medium antioxidant behavior. Subsequently, it was fractionated using 100% ethyl acetate as solvent. Its bio-water antimicrobial potential was evaluated using the microbial growth curve technique. *S. epidermidis* was used as pathogenic bacteria, and gentamicin was used as a positive control, which gave very positive results since inhibition rates of 91% were observed at a concentration of 0.3 ug/mL, values very close (96-98%) to those of the commercial antibiotic at concentrations of 40-0.04 μg/mL. Strain 145 was identified by the 16s rRNA technique as *Streptomyces parvulus*.

In compliance with this research project's second and third objectives, the factors affecting pigment production were evaluated and subsequently optimized. The result was a second-order polynomial model based on the Box-Behnken experimental design regression, whose optimal conditions were recorded at 30° C, 50 rpm, and seven days of incubation, producing 465.33 μg/mL. These conditions were validated in Bioreactor at a volume of 1 L, giving an error of 1.72%. Additionally, the R-square of 0.9006 indicated that the model obtained has reliability for producing the extracellular pigment of the *Streptomyces parvulus* strain. Finally, the depigmenting activity was evaluated, showing possible qualities at a concentration of 300 μg/mL and 10.94% tyrosinase inhibition. The results of the anti-inflammatory activity showed a significant difference between the control compound (Ibuprofen), the extract, and the pigmented fraction, in the inhibition of TNF-α and IL-10 interleukins. Therefore, it is determined that the fractionated and crude extract possesses anti-inflammatory properties. The disc diffusion technique qualitatively corroborates the anti-acne activity at a concentration of 10 μg/mL and 30° and 15 μL volume against *S. epidermidis*.

The orange-yellow extracellular pigment from *Streptomyces parvulus* meets the minimum bioactivity requirements to be considered safe. Additionally, it presents complementary functions such as depigmenting and anti-inflammatory, and excellent anti-acne potential.

Therefore, this study's extract and the pigmented fraction can be considered a possible cosmeceutical ingredient.

5.1 FINAL REMARKS

- Despite the extensive research that has been done on *Streptomyces* pigments, within what was reviewed in the literature, the application of this type of secondary metabolites in the cosmetic industry is minimal. A more excellent application in the food and textile industry was observed. Therefore, this research can be a pioneer in developing this field that invoices more than 80.73 billion dollars per year, with an estimated growth for 2025 of 177 billion dollars.

- *Streptomyces* strains from the Arauca rivers showed antimicrobial, cytotoxic, and antioxidant potential. This could be a future alternative if research on this genus continues to be developed so it can be postulated as a therapeutic ingredient in the cosmetic industry.

- Factors such as temperature, incubation time, and stirring speed significantly changed *Streptomyces parvulus* pigment production. Incubation time found stability and more significant influence in 7 days than at three days of fermentation. Although pigment production in the solid-state showed noticeable pigmentation after three days, liquid fermentation metabolized nutrients more slowly to achieve pigment production.

- The Plackett-Burman and Box-Benhken experimental designs performed well in obtaining the adjusted models to define the independent variables with the most significant positive effect on pigment production and their respective optimization conditions.

- The optimal conditions for *Streptomyces parvulus* extracellular pigment production were established at 30° C, 50 rpm, and seven days of incubation. The model was validated in the laboratory in a 1 L bioreactor, and 457 µg/mL of unfractionated pigment was obtained. The model error is only 1.72%.

5.2 SUGGESTIONS AND FUTURE RESEARCH

- Further purification or fractionation of the colored extract of the *Streptomyces parvulus* strain is recommended for future research. Because it is a highly polar extract, and its chemical configuration at the structural level is unknown. This could improve the results of the already evaluated bioactivities if pure bioactive fractions are obtained.

- It is proposed to analyze the nature of the compound by LC-HRMS technique, to have a more synthesized and precise reading of secondary metabolites that can explain the confirmed bioactivities.

- It is suggested to test the pigment in neutral cosmetic formulations and to evaluate bioactivities in In vivo and In vitro models. This can establish a possible entrepreneurship project, promoting the cosmetic industry's innovation and development in Colombia.

- It is recommended to analyze the chemical and physical stability of the pigment to determine if there is color migration if oxidation occurs if the bioactivity loses effectiveness, among other factors that would validate this pigment as a possible ingredient for the cosmetic industry.

- It is proposed for future research to elaborate detailed analyses similar to this one but change the experimental designs evaluated by mixtures to develop a synergic combination that potentiates the already considered bioactivities and those that can be tested related to cosmetics. To create a synergistic blend that potentiates the already evaluated bioactivities and can be tested and connected to cosmetics. This mixture could be patented.

- Finally, it is suggested to continue scaling the model adjusted to other volumes and evaluating additional factors, such as improvements in carbon and nitrogen sources.

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7 SUPPLEMENTARY FILES

Table 20 (S1)Gram Negative Bacteria evaluated in the antimicrobial test.

Gram Negative	Ref.
***Aeromonas* sp. (FPO6, FPO2)**	[40]
***Citrobacter* sp. FPO3, *Citrobacter freundii* (ATCC 8090)**	[40], [45]
***Edwardsiella* sp. FPO4**	[40]
Vibrio* sp. FPO5, *Vibrio cholerae	[40], [41], [67]
***Escherichia coli*, *E. coli* (ATCC.1330, ATCC 25922, ATCC8739, ATCC 8379, NIHJ JC-2, MTCC730)**	[15], [41], [45]–[47], [51], [52], [55]–[57], [60], [61], [63], [203], [204]
Proteus mirabilus	[41]
***Salmonella typhi*, *S. typhi* (ATCC6539, MTCC733), S. *paratyphae*, *Salmonella* sp., *S.enterica* MTCC1165, *S. enterica typhimurium* MTCC98,**	[41], [46], [51], [53], [56], [63], [204]
***Klebsiella oxytoca*, *K. pneumonia* (ATCC 1053, ATCC 13883, ATCC 13882, MTCC109), *K. pneumoniae*, *Klebsiella* sp.**	[15], [41], [45]–[47], [52], [55], [56], [58], [61], [63], [204]
P.aeruginosa* (ATCC 1074, ATCC 27853, ATCC 6538, ATCC9027), *P. aeruginosa	[15], [45]–[47], [51], [52], [56]–[58], [61], [203], [204]
***Shigella dysenteriae* (ATCC 1183), *Shigella* sp.**	[45], [46], [56], [204]
Serratia marcescens* (ATCC 14756), *S.marcescens	[45], [61]
Proteus* sp., *Proteus mirabilis*, *Proteus vulgaris	[46], [56], [204]

Table 21 (S2) Gram Positive Bacteria evaluated in the antimicrobial test.

Gram Positive	Ref.
***Mycobacterium smegmatis 3*, *Mycobacterium* tuberculosis H37Rv**	[48], [57], [59], [62]
***Staphylococcus aureus*, *S. aureus* (ATCC 1112, ATCC 25923, MTCC96, ATCC 29213, ATCC 6538, 209P JC-1, MTCC1430,15), *S. epidermidis*, MRSA, VRSA.**	[15], [41], [45]–[48], [51], [52], [55], [57], [59]–[61], [63], [204]
***Corynebacterium bovis* NIRD 129**	[59]
***Nocardia asteroides* 3318**	[59]
Bacillus* sp. FPO1, *B. cereus* (ATCC1015), *B. subtilis* ATCC6633, *B. cereus*, *B. subtilis*, *Bacillus typhi	[40], [46], [51], [52], [55], [56], [58], [60], [64], [203], [204]
Lactobacillus vulgaris	[41]
Enterococcus* sp, *Enterococcus faecalis* (ATCC 29212, MTCC9845), *Enterococcus faecalis	[46], [48], [56], [60], [61], [204]
***Micrococcus luteus* ATCC 379**	[43]
***Streptococcus pyogenes* ATCC19615, *Streptococcus mutans* MTCC890.**	[51], [58], [63]

Table 22 (S3) Mushrooms and Yeast evaluated in the antimicrobial test.

Fungi evaluated in the antimicrobial test	Ref.
Aspergillus niger* (PTCC 5011), *Aspergillus niger	[15], [45]
Candida albicans* (ATCC5027, ATCC 10231, ATCC 10259, TP-F0594), *Candida albicans	[15], [45], [46], [51], [57], [58], [60], [203]
***Penicillium citrinum* AS3.2788**	[203]
***Trichophyton rubrum* ATCC28188**	[203]
***Saccharomyces cerevisiae, Saccharomyces cerevisiae* TP-F0176**	[15], [60]
Alternaria solani	[15]
Bipolaris oryzae	[15]
Rhizoctonia solani	[15]
***Fusarium oxysporum, F.oxysporum* MTCC387**	[15], [57]

Table 23 Plackett-Burman design for evaluation of 6 factors influencing pigment production

		Factors					
		1	2	3	4	5	6
Experiments	1	+	-	+	-	-	-
	2	+	+	-	+	-	-
	3	-	+	+	-	+	-
	4	+	-	+	+	-	+
	5	+	+	-	+	+	-
	6	+	+	+	-	+	+
	7	-	+	+	+	-	+
	8	-	-	+	+	+	-
	9	-	-	-	+	+	+
	10	+	-	-	-	+	+
	11	-	+	-	-	-	+
	12	-	-	-	-	-	-
	Level	Incubation time	pH	Temperature	Concentration C	Concentration N	Agitation speed
	-1	7	6	25	Depends on	Depends on	150
	1	15	8	35	Depends on	Depends on	250

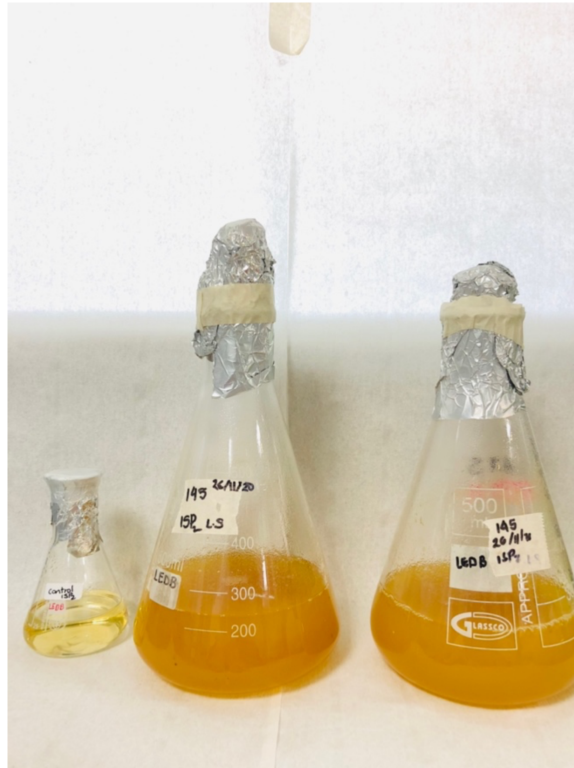
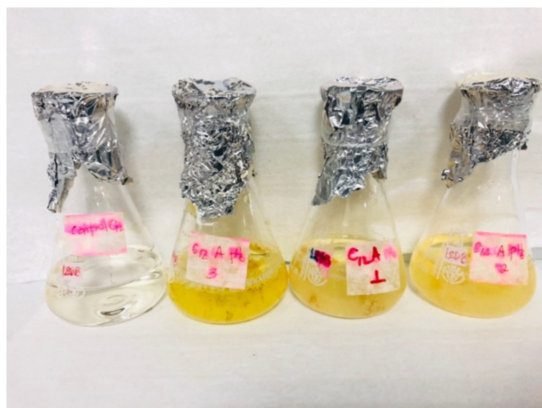


Figure 27 Fermentation of strain 145 at normal operating conditions (30 C°, seven days incubation, 150 rpm) in ISP2 culture medium with carbon source: glucose. Control medium ISP2 without inoculum, prepared at the same concentrations of carbon and nitrogen source, and subjected to the same conditions of pH, temperature, stirring speed and incubation time.

Figure 28 Evaluation of the different carbon sources to determine the most significant influence on pigment production in the shortest time, in the solid state, at 30° and 7 days of incubation. Control ISP2 (standard) without inoculum , prepared at the same concentrations of carbon and nitrogen source, and subjected to the same conditions of pH, temperature and incubation time.

A B



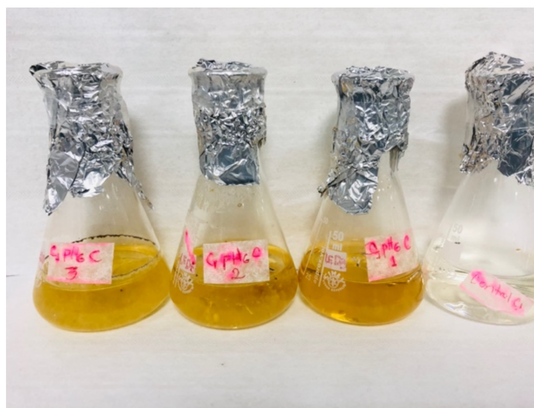


Figure 29 A). Plackett-Burman run #12, operating conditions: Incubation time= 3 days, pH=6, Temperature= 25° , 0.1% carbon source and 0.1% nitrogen source, rpm= 100, reported the lowest pigment concentration. B) Plackett-Burman run #1, operating conditions, Incubation time= 7 days, pH=6, Temperature=35°, 0.1% carbon source, 0.1% nitrogen source and rpm 100, reported the highest pigment concentration. Each run was inoculated with the same amount of biomass (30 mg). The control of each test was performed without inoculum and under the same concentrations of the corresponding runs.

A B

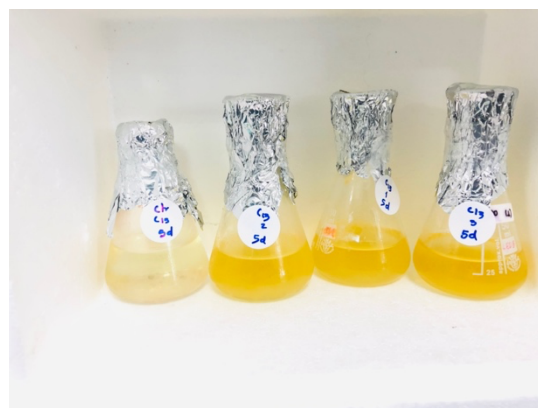


Figure 30 A) Run # 1 of the Box-Behnken experimental design at operating conditions: Temperature 30, incubation time nine days, and agitation 100 rpm; reporting the highest concentration (445 µg/mL). B) Run #13 of the experimental design, operating conditions: Temperature 35, incubation time five days, and agitation 150 rpm; reporting the lowest concentration (17.5 µg/mL). Each run was inoculated with the same biomass (30 mg). The control of each test was performed without inoculum and under the same concentrations of the corresponding runs.

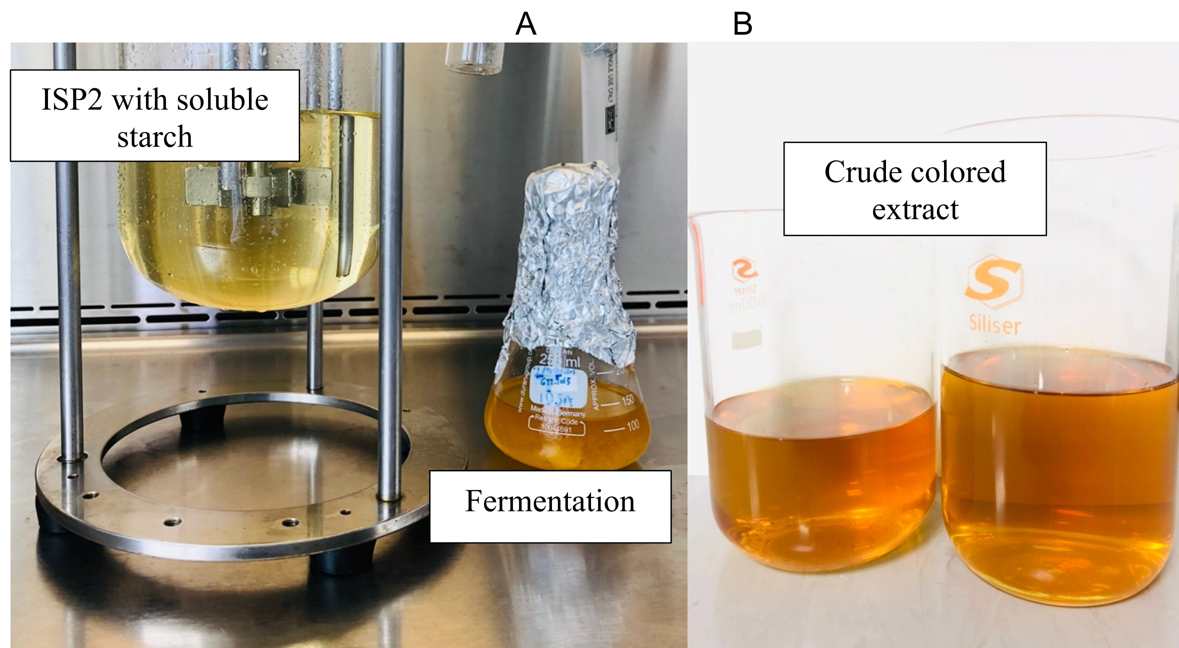


Figure 31 A) Validation of the model proposed by the Box-Benhkent experimental design in a bioreactor: at optimum conditions of 30, agitation 50 rpm, and seven days of incubation, with a standardized batch in biomass and scaled for 1 liter of fermentation (100 mL of inoculum). Control medium ISP2 with soluble starch and without inoculum. B) Obtaining the colored crude extract, centrifuged and free of biomass, reporting an unfractionated pigment production of 457 µg/mL.

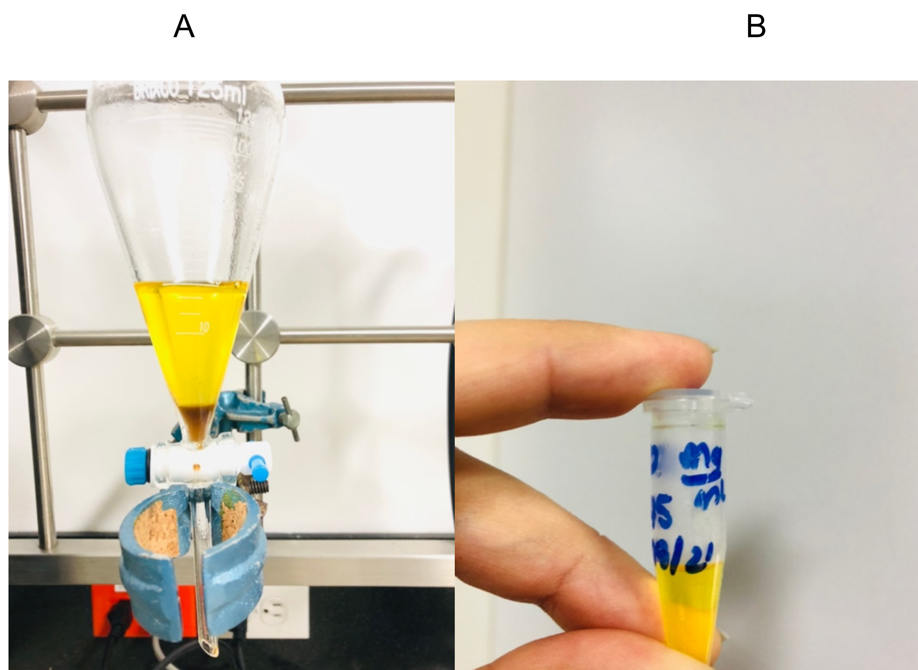


Figure 32 A) Fractionation of the crude extract at the optimal conditions of the validated model with 100% ethyl acetate in a decanter funnel. B) Obtaining the pigmented fraction at the validated optimum conditions after decantation. This was used to perform the respective bioactivity assays.