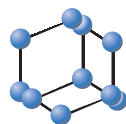


RESEARCH ARTICLE

BENTHAM
SCIENCE

Modeling and Statistical Optimization of Culture Conditions for Improvement of Antifungal Compounds Production by *Streptomyces albidoflavus* S19 Strain of Wastewater Origin



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Abstract: Background: The actinomycetes strains isolated from unexplored ecosystems are a promising alternative for the biosynthesis of novel antimicrobial compounds. Depending on the interesting antifungal activity of the studied strain S19, the statistical method seems to be an effective tool for optimizing the production of anticandidal molecules.

Introduction: This study was conducted in order to optimize the culture parameters (medium nutrients concentrations and initial pH value) affecting the production of antifungal metabolites from *S. albidoflavus* strain S19 (obtained from wastewater collected in Bejaia region, Algeria) using Response Surface Methodology (RSM). The best conditions for anti-*Candida albicans* compounds biosynthesis were determined.

Methods and Results: The antimicrobial producer strain *S. albidoflavus* S19 was identified on the basis of morphological, chemical characters and physiological characteristics along with 16S rRNA gene sequencing analysis.

Response Surface Methodology by Central Composite Design (CCD) was employed to improve the anti-*C. albicans* agents production through the optimization of medium parameters. The highest antifungal activity was obtained by using a mixture of 2g l⁻¹ starch, 4g l⁻¹ yeast extract, 2g l⁻¹ peptone at pH 11.

Conclusion: The strain S19 isolated from wastewater showed a significant anti-*C. albicans* activity and this study revealed the effectiveness of RSM and CCD for increasing bioactive compounds production, rising the diameter of inhibition zones from 13 to 34 mm.

Keywords: *Streptomyces albidoflavus*, central composite design, RSM, optimization, antifungal production, antimicrobial activity, *Candida albicans*.

1. INTRODUCTION

The increase of pathogens antibiotic resistance, the lack and the pharmacological restrictions of antibiotics availability are barriers that require the discovery of new antimicrobial substances. About 100 antibiotics have been marketed to treat human, animal and plant diseases [1].

Candida albicans is a common fungal pathogen responsible for several human fungal infections [2]. Immunocomp-

romized patients, such as AIDS or neutropenic patients, are particularly high-risk for *C. albicans* infections that can become systemic [2, 3]. Among the diverse marketed drugs, antifungals represent a restricted class having an important role in controlling fungal infections. Recently, the need for new and effective antifungals is a major challenge for the pharmaceutical industry, particularly with the increase of opportunistic infections in immunocompromised patients [4]. It has been proven that natural sources are at the origin of the discoveries of new drugs [5]. Many compounds with highly biological activity have been extracted from microorganisms, plants and animals [1]. This implies the screening of microorganisms and plant extracts [6] but unfortunately, the search

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for new, more efficient, broad-spectrum antifungals progressed slowly [7]. Actinomycetes are Gram-positive bacteria including branching unicellular microorganisms [8]. They are extensively used in industries resulting from their aptitude to manufacture numerous antibiotics [9], anticandidal [10], or anti-inflammatory compounds [11], enzymes, vitamins, growth hormones and anti-cancer agents [12]. Members of *Streptomyces* genus can produce diverse active compounds with antimicrobial properties, developed for pharmaceutical uses [13]. Indeed, this genus accounts for the synthesis of about 60 % of commercialized antibiotics [1]. In addition, valuable metabolites are also produced such as enzyme inhibitors, enzymes (lipases, cellulases, amylases and proteases) [14]. Therefore, *Streptomyces* are commonly investigated [15, 16], in particular from underexplored ecosystems, for new drugs discovery.

For the majority of antibiotics, including those recently marketed, fermentation is the most common procedure for bioactive compounds' production as a consequence of the complexity and the high cost of chemical synthesis processes. Thereby, enhancing and improving microbial strains culture conditions remain the main tools for reducing volumes production and costs and guarantee quality and reproducibility of the drug production [6]. Secondary metabolites biosynthesis is significantly influenced by the strain and these cultural conditions [17]. Indeed, Parekh *et al.* [18] reported the impact of culture parameters (nature and concentration) on antibiotic production. So, carbon and nitrogen sources as well as minerals are highly required in the metabolism not only for the growth but also for the antibiotic production by *Streptomyces* strains [19, 20] and numerous studies showed the importance of the optimization for the metabolites production [21, 22]. Furthermore, Wang *et al.* [23] stated that the metabolic profile along with the kind and the amount of secondary metabolites production is highly affected by minor modifications in culture medium composition. So, in order to enhance the production of target metabolites, optimization of culture medium is required. One factor at a time (OFAT) approach was the main used procedure which is however time-consuming, claiming high number of experiments [24]. Consequently, statistical experimental design approach and response surface methodology (RSM) are widely applied to determine the most significant factors and their optimal levels affecting the production [23, 24]. RSM with Central Composite Design (CCD) is used for selected factors optimization [17, 23, 24].

Hence, the proposed study is an effort to screen the actinomycetes isolated from wastewater in Bejaia region (Algeria), as an unexplored diversity ecosystem. This diversity can be explored for isolation and characterization of native actinomycetes for antimicrobial metabolites, essentially displaying antifungal properties. In order to optimize the medium for the production of anticandidal compounds, the design of experimental methods was performed.

2. MATERIALS AND METHODS

2.1. Actinobacteria Strain Isolation

The actinomycete strain designated S19 was isolated from a sample collected from wastewater treatment plant of

Bejaia region in Algeria. Serially diluted suspensions up to 10^6 were prepared and spread (0.1 ml) onto Starch-Casein-Agar (SCA) medium [25] supplemented with $K_2Cr_2O_7$ ($50 \mu g ml^{-1}$) to inhibit the development of fungi. The plates were incubated for four weeks at 28°C. Based on the morphological characteristics such as aerial and substrate mycelia colour on the different tested media, sporulation according to the International *Streptomyces* project (ISP) [26] and its broad spectrum of antimicrobial activity (against both Gram positive and negative bacteria and *C. albicans*), the actinomycete isolate S19 was selected and purified on SCA medium, and then preserved at -20°C in 20% glycerol.

2.2. Characterization of Strain S19

The morphological and cultural characteristics such as the colour of aerial and substrate mycelia and also pigmentation, were noted from 7-14 days-old cultures grown at 28°C on the following ISP agar media: Yeast extract-tryptone (ISP 1), Yeast extract-malt extract (ISP 2), Oatmeal (ISP 3), Inorganic salts-starch (ISP 4), Glycerol-asparagine (ISP 5), Peptone-yeast extract-iron (ISP6), Tyrosine-asparagine (ISP 7) and YEGA [27]. For cell shape, arrangement for undisturbed hyphae, length of hyphae, branching pattern, reproductive structure and chains of spores, 7-14 days incubated cultures at 28°C on SCA medium were observed under the optic microscope with magnification up to x400.

The chemical examination of the diaminopimelic acid (DAP) isomers and whole-cell sugar were determined as described by Seong *et al.* [28] and Lechevalier and Lechevalier [29] respectively.

The biochemical characteristics of strain S19 were, in essence, based upon the assimilation of various carbohydrates and amino acids as unique carbon and nitrogen source respectively, according to Pridham and Gottlieb [30], decomposition of xanthine as described by Locci [31], degradation activity of several organic compounds as gelatin liquefaction, hydrolyzation of casein and tyrosine, utilization of acetate, citrate and oxalate as described by Gordon *et al.* [32], starch hydrolysis and reduction of nitrate according to Marchal *et al.* [33]. The lipasic activity was tested using Tween 80 according to Sierra [34]. All the results of the tests are noted after 7-14 days of incubation at 28°C.

For physiological growth characteristics, the strain S19 inoculated on SCA plates was incubated for 7-10 days at different temperatures (28, 30, 37 and 45°C), different pH values (3, 5, 7, 9 and 11 with an interval of 0.2 units) and different NaCl concentrations (1, 1.5, 2.0, 2.5, 3, 3.5, 4, 4.5 and 5 mol l⁻¹). Moreover, sensitivities to phenol (at 0.05 and 0.1%), sodium azide (at 0.001 and 0.01%), potassium tellurite and five heavy metals (50 mg ml⁻¹ of Cd₃N₂O₆, CuSO₄, FeN₃O₉, K₂Cr₂O₇, and ZnSO₄), as described by Daboor *et al.* [35], were evaluated on SCA medium after 7-10 days of incubation at 28°C.

2.3. The 16S rRNA Gene Amplification and Phylogenetic Analysis

A preculture of 50 ml of ISP medium was prepared with a single colony of the strain in a 250 ml flask, which was incubated under agitation at 28°C for 2 days. The genomic DNA

used for PCR amplification was extracted with Wizard[®] Genomic DNA Extraction Kit from Promega Corp. and the concentration of the DNA used was determined using a NanoDrop[™] Lite spectrophotometer (Thermo Scientific, Villebon sur Yvette, France). The 16S rDNA was amplified using the PCR method with Taq DNA polymerase (Master Mix x2) and universal primers S1 (5'-AGAGTTTGATC(A,C)TGGC-TCAG -3') and S2 (5'-GG(A,C)TACCTTGTTACGA (T,C)TTC-3'). Amplification was carried out in 50 µl reaction mixture containing 25 µl of Master Mix (Thermo Fisher Scientific) DreamTaq polymerase, 2.5 µl (20 µmol l⁻¹) of each primer and 20 µl of genomic DNA. The determined sequences were compared with the GenBank databases using the basic local alignment search tools (BLAST) software provided online by the National Center for Biotechnology Information (Bethesda, MD, USA). Analyses were repeated at least three times. Sequence data were aligned with CLUSTAL_W [36] and the phylogenetic tree was constructed using MEGA 6.0 with the neighbor-joining method algorithm and 1000 bootstrap resampling interaction [37].

2.4. Selection of the Best Medium for Antibiotic Biosynthesis

The cylinder agar assay [38] was investigated to determine the antimicrobial activity of strain S19 against Gram-positive bacteria (*Micrococcus luteus*, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, MRSA ATCC 43300 and *Listeria innocua* CLIP 74915), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhi* ATCC 14028) and a yeast (*Candida albicans* ATCC 10231). Strain S19 was grown on Starch-casein-agar (SCA), Mincer (M) [39], Mincer supplemented with 50% seawater (MSW), Gause (G) [40], Gauss supplemented with 50% seawater (GSW), Czapeck Dox agar (Cz) [41], Czapeck supplemented with 50% seawater (CzSW), ISP 1 and ISP 2 media. The cultures were incubated at 30°C for 7 days and 6 mm diameter agar disks of the actinobacterial culture were prepared by using sterile Cork borers and aseptically transferred to Muller-Hinton (or Sabouraud dextrose for yeast) plates having 10⁷ CFU ml⁻¹ of the target microorganism. After at least 2 hours of diffusion of any antibiotic produced at 4°C, the dishes are incubated at 37°C and the diameters of the inhibition zones were determined after 18 to 24 h.

2.5. Optimization of Antifungal Production Using CCD and RSM

The number of experiments, time and material resources can be decreased using experimental design which is widely used for controlling the effects of parameters in many processes. Several experimental designs have been considered for studying such models.

In this study, Central Composite Design (CCD) was used to optimize four operating factors, namely starch concentration (X₁), yeast extract concentration (X₂), peptone concentration (X₃) and pH of the medium (X₄) on the biosynthesis of the anti-*C. albicans* compound after the study of their effect. The latter led to obtain a quadratic model with 2⁴ factorial trails, eight star points to estimate quadratic effects and six central points to fit the second-order polynomial model. 30 experiments were required in this design where each fac-

tor was studied at five experimental levels: -α, -1, 0, +1, +α, with α = 2^{n/4}, where n is the number of variables and 0 corresponded to the central point of each factor.

The levels of factors used for experimental matrix are given in Tables 1 and 2, respectively. The anti-*C. albicans* activity was tested for antibiotic production after 7 days of growth at 30°C. All the experiments were carried out in triplicate and the experimental results of CCD were fitted via the response surface regression procedure, using the following second order polynomial equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad i = 1, 2, 3, \dots, k \quad (1)$$

Table 1. Values and levels of operating parameters.

Factors	Levels				
	-α (-2)	-1	0	+1	+α (+2)
X ₁ : Starch concentration (g l ⁻¹)	2	6	10	14	18
X ₂ : Yeast extract concentration (g l ⁻¹)	2	3	4	5	6
X ₃ : Peptone concentration (g l ⁻¹)	0.5	1.25	2	2.75	3.5
X ₄ : pH	3	5	7	9	11

Where *Y* is the predicted response, β₀ is the regression coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient and X_i is the coded level of independent variables.

The antifungal production yield is the dependent output response variable which is expressed as the diameter of inhibition zone against *C. albicans* (mm). The independent variables are coded for statistical calculation according to the following equation:

$$x_i = \frac{(X_i - \bar{X}_i)}{\Delta X_i} \quad i = 1, 2, 3, \dots, k \quad (2)$$

where x_i is the coded value of the ith variable, X_i is the original or real value, \bar{X}_i the value of X_i at the center point of the investigated area and ΔX_i is the step size.

3. RESULTS

3.1. Strain S19 Isolation

A total of 156 actinomycete isolates were obtained from a wastewater sample from Bejaia (Algeria). This ecosystem is unexplored for the isolation of actinomycetes. It was selected for its abundance of organic matters and organisms that are competitor for nutrients.

Among the strains isolated and tested for their ability to produce antimicrobial compounds, S19 showed the largest activity spectrum against the tested microorganisms (data not shown). So, this strain was selected for further study.

3.2. Characterization and Taxonomy of Strain S19

Significant growth of aerial mycelium of the actinomycetal strain S19 added to good sporulation was observed on six

tested media, whereas no sporulation was observed on ISP 1 and ISP 5 media. Melanoid pigments were not produced on ISP 6 and ISP 7 media where grayish-green soluble pigment was produced on ISP 3 medium. Growth, aerial and substrate mycelia colour, sporulation and soluble pigment production

of strain S19, after incubation at 28°C for 7-14 days are summarized in Table 3 and the light-microscopic observation of S19 (10x40 magnification) after 14 days of culture on ISP 4 medium is shown in Fig. (1).

Table 2. Central composite design (CCD) of factors in coded value for optimization of the antifungal activity yield.

Run No.	Natural Values of Parameters				Coded Values of Parameters					Observed Antifungal Activity y (mm)
	X_1 (g l ⁻¹)	X_2 (g l ⁻¹)	X_3 (g l)	X_4	x_0	x_1	x_2	x_3	x_4	
1	6	3	1.25	5	1	-1	-1	-1	-1	13.95
2	14	3	1.25	5	1	1	-1	-1	-1	17.64
3	6	5	1.25	5	1	-1	1	-1	-1	0
4	14	5	1.25	5	1	1	1	-1	-1	17.53
5	6	3	2.75	5	1	-1	-1	1	-1	19.55
6	14	3	2.75	5	1	1	-1	1	-1	12.06
7	6	5	2.75	5	1	-1	1	1	-1	0
8	14	5	2.75	5	1	1	1	1	-1	16.83
9	6	3	1.25	9	1	-1	-1	-1	1	12.56
10	14	3	1.25	9	1	1	-1	-1	1	9.02
11	6	5	1.25	9	1	-1	1	-1	1	25.42
12	14	5	1.25	9	1	1	1	-1	1	0
13	6	3	2.75	9	1	-1	-1	1	1	20.88
14	14	3	2.75	9	1	1	-1	1	1	14.45
15	6	5	2.75	9	1	-1	1	1	1	18.16
16	14	5	2.75	9	1	1	1	1	1	9.23
17	10	4	2	7	1	0	0	0	0	13.10
18	10	4	2	7	1	0	0	0	0	13.50
19	10	4	2	7	1	0	0	0	0	13.18
20	10	4	2	7	1	0	0	0	0	13.00
21	10	4	2	7	1	0	0	0	0	13.68
22	10	4	2	7	1	0	0	0	0	13.7
23	2	4	2	7	1	-2	0	0	0	14.02
24	18	4	2	7	1	2	0	0	0	10.66
25	10	2	2	7	1	0	-2	0	0	15.50
26	10	6	2	7	1	0	2	0	0	12.92
27	10	4	0.5	7	1	0	0	-2	0	9.3
28	10	4	3.5	7	1	0	0	2	0	14.6
29	10	4	2	3	1	0	0	0	-2	0
30	10	4	2	11	1	0	0	0	2	17.80

Table 3. Morphological features of strain S19 on different media.

Media	Mycelium Growth	Aerial Mycelium Colour	Substrat Mycelium Colour	Spores	Soluble Pigment
ISP 1	++	White	White	No spore	Absent
ISP 2	+++	White-grayish	White	Grey	Absent
ISP 3	+++	Light greenish	Grayish green	Light grayish	Grayish green
ISP 4	+++	White	White	Yellow	Absent
ISP 5	++	Grayish-white	White	Gray	Absent
ISP6	+++	Grayish-white	White	Gray	Absent
ISP7	+++	White	White	Grayish- white	Absent
YEGA	+++	Grayish white	White	Gray	Absent

ISP: International *Streptomyces* Project medium. +: poor; ++: moderate; +++: good.

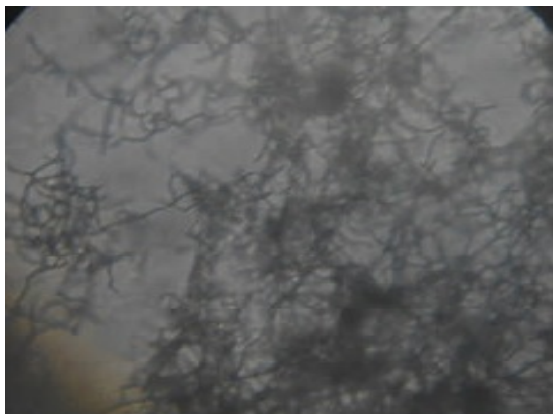


Fig. (1). Light microscopic observation of isolate S19 after 14 days of culture on ISP 4 medium (40x 10 magnifications).

The chemotaxonomic study of strain S19 showed the presence of LL-DAP in the peptidoglycan and glycine in the cell wall. The whole-cell hydrolysate did not contain characteristic sugars, typical of cell wall type IC [42].

Based on the morphological and chemotaxonomic features, the isolate S19 was found to be a member of the genus *Streptomyces*.

3.3. Phylogenetic Characteristics

The 1,489 nt 16S rDNA sequence of the strain S19 has been deposited in the GenBank data library and has been assigned the accession number KT996127. This sequence was aligned with those of *Streptomyces* reference species available in the GenBank database indicating that S19 was related to the genus *Streptomyces* (similarity level between 96 and 99%). Its position in the 16S rDNA *Streptomyces* tree is shown in Fig. 2. This isolate has 99% similarity level with *S. albidoflavus* (AJ002090) [43], the most closely related species.

The physiological and biochemical properties of strain S19 are summarized in Table 4. Isolate S19 growth occurs between pH 3 and pH 11 with an optimum at pH 9. The strain is also mesophilic with a growth optimum at 30°C; it

assimilates 15 of the tested carbon sources, Sodium acetate and citrate but not inositol nor sodium oxalate. This isolate degrades starch, Tween 80, tyrosine, casein from milk, utilizes all tested nitrogen sources and liquefies gelatin. Moreover, it is not sensitive to the five tested heavy metals.

Table 4. Physiological and biochemical characteristics of strain S19.

Characteristics	Results	Characteristics	Results
Range of temperature	25-45°C	Nitrogen source utilization	
Optimum temperature	30°C	Alanine	+
Range of pH for growth	3-11	Arginine	+
Optimum pH	9	Asparagine	+
Growth in the presence of NaCl	1-2 mol l ⁻¹	Aspartic acid	+
Carbon source utilization		Cysteine	-
Arabinose	+	Diphenylamine	-
Adonitol	+	Glutamic acid	+
Galactose	+	Glycine	+
Glucose	+	Histidine	+
Lactose	+	Isoleucine	+
Levulose	+	Leucine	+
Maltose	+	Methionine	+
Mannose	+	Ornithine	+
Raffinose	+	Phenylalanine	+
Ribose	+	Proline	+
Saccharose	+	Serine	+
Salicin	+	Threonine	+
Sorbitol	+	Tryptophan	+
Threalose	+	Tyrosine	+

Xylose	+	Valine	+
Inositol	-	Xanthine	-
Hydrolysis of:		Assimilation of sodium:	
Starch	+	Acetate	+
Tween 80	+	Citrate	+
Liquefaction of Gelatin	+	Oxalate	-
Tyrosine	+	Resistance to:	
Casein from milk	+	Phenol (0.01%)	+
		Potassium tellurite (0.01%)	+
		Cadmium	+
		Chromium	+
		Copper	+
		Iron	+
		Zinc	+

+: positive test, -: negative test.

Based on the genotypic result, strain S19 belongs to the species *S. albidoflavus*. However, this strain could be distinguished from *S. albidoflavus* DSM 40455^T by some phenotypic properties such as sporulation on some media, degradation of raffinose and colour of soluble pigment on ISP 3 medium. Strain S19 produces aerial spore mass at 40-45°C, while strain DSM 4055^T is unable to produce this mass at 40°C.

3.4. Selection of Basic Medium for the Antimicrobial Activity of Strain S19

The nature of habitat of the microorganisms as well as the differences in the composition of the substrate affects significantly their antimicrobial activity. In addition, the strain and the target microorganisms can also have an influence. Eleven media: standards (ISP 1, ISP 2, M, SCA, G and Cz) or added with 50% of seawater (ISP 2-SW, M-SW, SCA-SW, G-SW and Cz-SW), reported in literature for production of various secondary metabolites, were investigated to determine the optimal nutrient medium for antibiotic biosynthesis by strain S19. The medium selected for further study is the one on which the isolate exhibited the largest antibiotic production spectrum against the tested microorganisms.

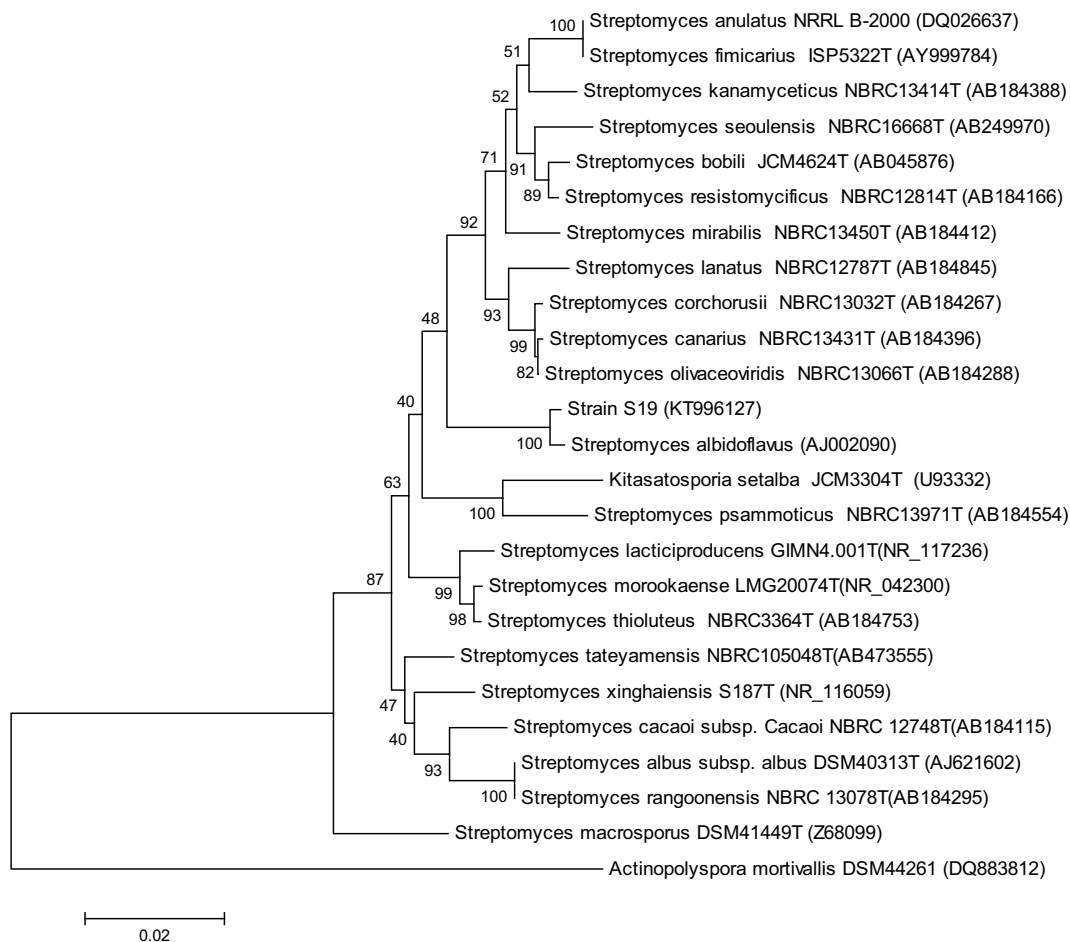


Fig. (2). Neighbour-joining tree based on 16S rDNA sequence showing the relations between strain S19 and type species of the genus *Streptomyces*. The numbers at the nodes indicate the levels of bootstrap support based on Neighbour-joining analysis of 1,000 resampled datasets. Bar, 0.02 nt substitution per nt position. *Actinopolyspora mortivalis* DSM 44261 (DQ883812) has been used as outgroup.

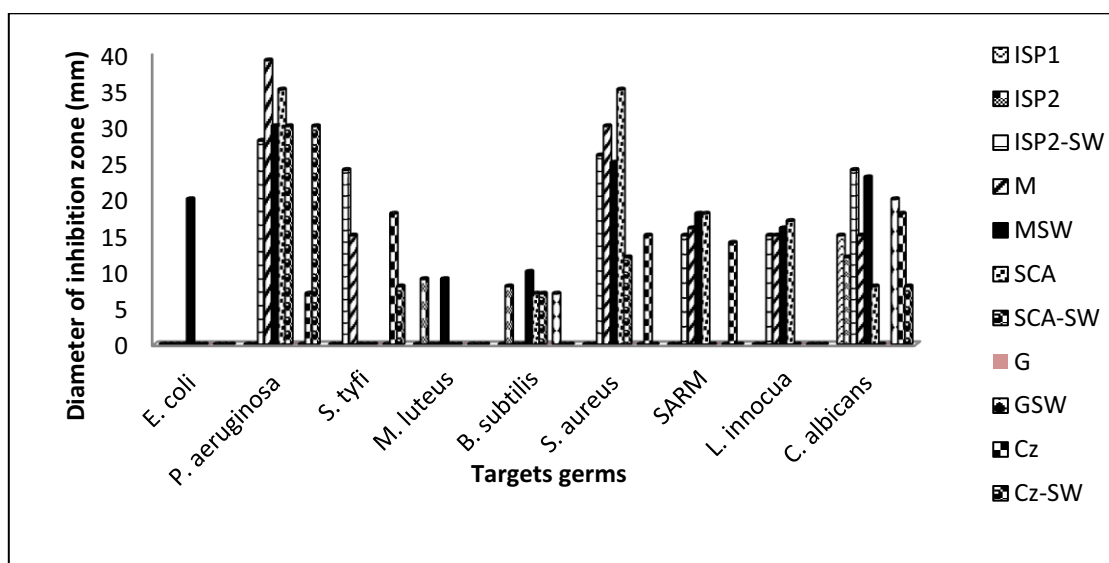


Fig. (3). Comparison of the effect of culture media composition on bioactive metabolite production by strain S19.

According to the results depicted in Fig. 3, all tested media exhibited an impact on the antimicrobial compounds production by strain S19. On M-SW, this strain was able to produce the largest spectrum of bioactive metabolites against all tested microorganisms. Indeed, by comparing antimicrobial activities of the isolate S19 against *E. coli*, *P. aeruginosa*, *M. luteus*, *B. subtilis*, *S. aureus*, MRSA, *L. innocua* and *C. albicans* on the tested media, culture on M-SW presented growth inhibition diameters of 20, 30, 9, 10, 25, 18, 16 and 23 mm, respectively. Based on these results, M-SW was selected to optimize the production of antifungal metabolites by strain S19 using CCD and RSM.

3.5. Optimization of Antifungal Production Using CCD and RSM

In this study, 30 experiments were carried out to obtain optimal combinations of starch concentration (X_1), Yeast-extract concentration (X_2), Peptone concentration (X_3), and initial pH (X_4) for antifungal production. By applying multiple regression analysis to experimental data of central composite design (CCD) shown in Table 1, the effect of each factor and their second order interactions of S19 strain against *C. albicans* is explained by a second order polynomial model.

Table 2 summarizes the results of second-order CCD experiments. The model coefficients (Eq 1) are estimated by the standard least-squares regression method using the "EXCEL" software. Three statistical tests are necessary to evaluate the adequacy of the model, namely the Student's *t*-test for the significance of the factors, the *R*-squared test and Fisher's tests.

After the 30 experiments (Table 2) and the elimination of non-significant effects, the model equation obtained was as follows:

$$\hat{Y} = 13.36 - 0.85x_1 - 1.58x_2 + 1.07x_3 + 1.99x_4 + 0.86x_{12} - 4.68x_{14} - 0.78x_{23} + 1.54x_{24} + 1.03x_{34} + 0.4x_2^2 - 0.17x_3^2 - 0.93x_4^2 \quad (3)$$

Where \hat{Y} is the predicted antifungal yield, X_1 is starch, X_2 is yeast extract, X_3 is peptone concentration and X_4 is initial pH.

The observed response variability values related to the experimental factors and their interaction is determined by the values of the coefficient of determination (R^2) [44].

According to the obtained value of the determination coefficient (R^2) of the model which was found to be 0.947, the variability of the response could be explained by the model at 94.7%.

According to Chen *et al.* [45] the R^2 value greater than 0.9 is required for a very high correlation of the regression model. Indeed, the R^2 obtained value indicates a good correlation between the observed and predicted responses and suggests that the model is reliable for antifungal production.

The obtained regression equation (Eq. 3) shows that concentrations of starch (x_1), yeast extract (x_2), peptone (x_3) and pH (x_4) all have an individual influence on the anti-*C. albicans* metabolite production. The pH (x_4) has the strongest effect on the response since coefficient of x_4 ($b_4 = +1.99$) is larger than the coefficients of the other investigated factors. A positive sign of this coefficient indicates that the antifungal production is enhanced at high pH or alkaline media. The order for factors strength on antifungal production following pH was found as yeast extract concentration (x_2) with a negative effect ($b_2 = -1.58$), peptone concentration (x_3) with a positive influence ($b_3 = +1.07$) and finally starch concentration (x_1) with a negative effect ($b_1 = -0.85$).

The significance interactions found by the design of experiments in order are essentially between the concentrations of starch and pH (x_1x_4) ($b_{14} = -4.68$), concentrations of yeast extract and pH (x_2x_4), peptone concentration and pH (x_3x_4), starch and yeast extract (x_1x_2) and between concentrations of yeast extract and peptone (x_2x_3).

The corresponding 2D and 3D dimensional response surfaces of the quadratic model are shown in Figs. 4a and b. The figures are drawn in starch concentration (x_1) – pH (x_4) plan

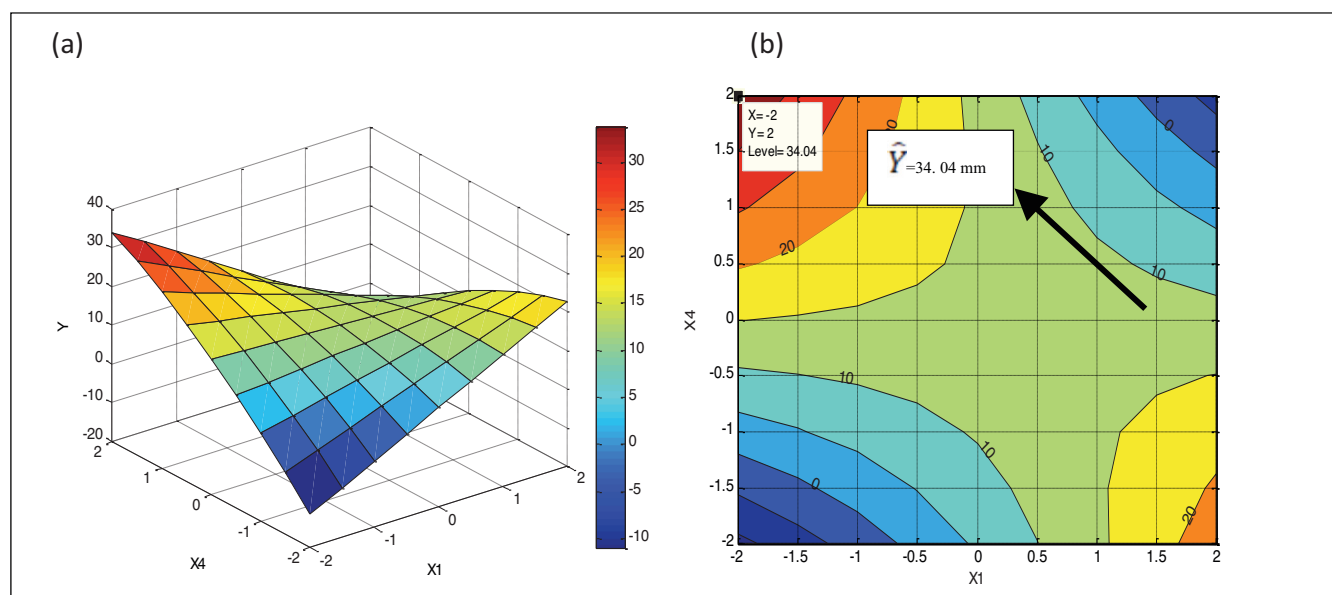


Fig. (4). 2D and 3D Response surface of antifungal activity against *C. albicans*.

(the most important interaction affecting the response) for $x_2 = 0$ and $x_3 = 0$ using “MATLAB 7.0” software. The analysis of these figures showed that the maximum predicted diameter of inhibition zone against *C. albicans* (34 mm) is reached by decreasing the starch concentration and increasing the pH as indicated by the row in Fig. (4a). The corresponding coordinates of this optimum are as follows: starch concentration = 2 g l^{-1} ($x_1 = -2$), yeast extract = 4 g l^{-1} ($x_2 = 0$), peptone = 2 g l^{-1} ($x_3 = 0$) and pH = 11 ($x_4 = 2$).

4. DISCUSSION

In the way of actinomycetes isolation from underexplored sources, an antimicrobial producer, *Streptomyces albidoflavus* S19, isolated from wastewater of Bejaia region in Algeria, was selected. This strain exhibited important antimicrobial activities against five species of Gram-positive bacteria, especially against MRSA and *S. aureus*, three Gram-negative bacteria especially *P. aeruginosa* and *S. typhi*, and the yeast *C. albicans*.

Sarmiento-Vizcaino *et al.* have reported cultivable *S. albidoflavus* strains to be ubiquitous in most studied habitats (aerial, terrestrial, and marine) in a broad geographic region (Northern Spain, Northern Portugal and South of France, from 2008 until 2012) [46]. In the past few years, Actinobacteria isolated from several extreme environments and unexplored habitats around the world have been intensively studied. So, the exploration of particular ecosystems (temperature, pH, aeration or osmotic stress) promotes detection of actinomycetales bacteria which may optionally have a potential antibacterial and/or significant antifungal activity [47]. In addition, current research of novel molecules of antibiotics is directed towards the isolation of strains from specific ecosystems and hostile environments previously untapped [48].

Several genera affiliated with the Actinomycetales order are the source of various drugs used against common diseases

and members of the genus *Streptomyces* are the main producers of bioactive molecules widely used in different biotechnology industries [49].

In fact, in current research programs; previously unexplored natural habitats are behind the isolation of various *Streptomyces* strains producing several antimicrobial agents [50, 51].

The results of the studies on morphological, chemotaxonomic, physiological characteristics and the phylogenetic analysis indicated that strain S19 had a high concordance with *S. albidoflavus* DSM 40455^T strains described by Rong *et al.* [43], and a high similarity (99%) with its 16S rDNA sequence. However, some peculiar cultural properties are noted such as the colour of substrate mycelium on ISP 5 and ISP 6 media, the spore colour of strain S19 on ISP 4 medium, and the soluble pigment produced on ISP 3. For the physiological differences, raffinose is assimilated by strain S19 while this carbon source is not utilized by the type-strain *S. albidoflavus* described by [43]. The isolate S19 tolerates saline conditions up to 11% of NaCl and a wide range of pH (between pH 3 and pH 11) and temperatures (28-45°C), in agreement with previous reports [46, 52].

The influence on antibiotic production of *S. albidoflavus* S19 strain by culture medium was investigated and among eleven media reported for production of secondary metabolites, M-SW was selected because it allowed the largest spectrum of antibiotic production by this strain, thereby optimizing the production of anti-*C. albicans* compound. The composition of the growth medium has a relevant influence on metabolite production [53]. There is no universal medium applicable to all microorganisms; however, Starch Casein Agar medium (SCA) was reported to be the best one for antifungal production by *S. albidoflavus* PU 23 [54].

The combination of CCD design with RSM for optimizing the antifungal production by *S. albidoflavus* S19 against

C. albicans is an effective and reliable tool to select the statistically significant factors and to find the optimal concentration and levels of those factors in the culture medium. Out of the four studied factors, pH and peptone concentration were found to have a positive influence on antifungal production, whereas the concentration of starch and yeast extract had a negative influence. Concerning the effect of pH, the results of this study seem opposite to those reported in the literature. Augustine *et al.* showed that the optimal pH for antifungal production by *S. albidoflavus* strain PU 23 was equal to 7 [54]; the same result was reported for *S. albidoflavus* strain ANU 2677 [55], and a value of this factor greater than 8 was unfavourable for antibiotic production. This value is probably strain-dependent.

The negative effect of starch concentration could be explained by the catabolite repression from its higher concentration [56]. The regulation of the rate of carbon consumption in *Streptomyces* seems to be unique; nonetheless, the catabolic mechanism in this genus has not been unraveled [57]. In particular, starch is the preferred carbon source by the majority of *Streptomyces* for the production of secondary metabolites [56, 58, 59]. Thus, it is not unexpected that starch is a significant factor, as shown by the results presented here. However, in this study, the anti-yeast production by the strain S19 decreases with the increasing of starch concentration. According to carbon sources, cell growth and secondary metabolite production in *Streptomyces* spp. are strongly related to species-specific variation [60]. In the complex nitrogen sources, yeast extract supports the greatest mycelium biosynthesis and favours a rapid growth [61]. Previous experiments have shown that it is the preferred nitrogen source of *Streptomyces* strains [62-64]. Moreover, Himabindu and Jetty found that yeast extract is favourable for growth but not for antibiotic production [65].

In this study, as shown by the second order equation developed, yeast extract is an important factor with a negative effect which means that it may decrease the anti-*Candida* molecule synthesis.

The interaction of starch with pH was the most important one. From CCD, the most optimal condition for anti-*C. albicans* production was found to be pH 11, 2 g l⁻¹ of starch, 4 g l⁻¹ of yeast extract and 2 g l⁻¹ of peptone. In addition, validation of the model suggested, unequivocally, the reliability of RSM for optimization of media for antibiotic production.

CONCLUSION

The results of this study suggest that wastewater Actinobacteria are a particular group of microorganisms that can provide new metabolites.

The isolation of strains with antimicrobial activity suggests that wastewater may be an ecological niche, harbouring largely untapped microbial diversity and untapped potential for new secondary metabolites. The results obtained from this work are promising and deserve to be studied further as the kinetic production of the anticandidal compound(s), the purification, characterization and identification of the active secondary metabolites produced by *S. albidoflavus* strain S19.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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