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Novel Research on Rapamycin Analogue Production by *Streptomyces* sp. Strain RHS5

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Abstract

Rapamycin (Rap), as a macrocyclic polyketide with immune suppressive, antifungal, and anti-tumor activity produced by *Streptomyces hygroscopicus*, is receiving considerable attention for its significant contribution in medical field. However, the production capacity of the wild strain is very low. Due to the high price of rapamycin drug (1MG 30/TAB price= 900PT) and the limited number of producing organisms, the present research was conducted to search for novel strains from local environments capable of producing rapamycin. *Streptomyces* sp. RHS5 selected from thirty-three actinobacterial isolates was the most potent in producing the active metabolite and was compared with rapamycin produced by *Streptomyces hygroscopicus* using bioautography. The chemical structure of the purified compound determined by spectroscopic analyses was found to be analogue to rapamycin. So far, considerable efforts have been made to improve rapamycin production, such as fermentation technology optimization. In this research, the production of rapamycin analogue was economically achieved by applying the solid-state fermentation (SSF) using wheat bran as a substrate and optimization of culture condition was achieved by the statistical design Plackett- Burman. The productivity of rapamycin analogue after optimization increased by 1.18 fold compared to basal medium.

Keywords
Streptomyces sp. RHS5, Rapamycin, Solid state fermentation, Statistical optimization.

Introduction

Rapamycin (Rap) is a lipophilic macrolide and a natural secondary metabolite produced by *Streptomyces hygroscopicus* [1] in Rapa-Nui (Easter Island) isolated from a collected soil sample. Rap was first reported as antifungal agent (mainly against *Candida* spp.) and has medical application as as antitumor, immunosuppressive [2, 3], anti-aging [4, 5] and effectiveness in treatment of Parkinson's disease [6]. It performs antitumor activity on a tumor cell by hindering its proliferation and triggering apoptosis [7]. Rapamycin has lower toxicity and greater activity which is 150 times as that of cyclosporine [8]. From the American FDA, Rap has two approvals, the first in kidney transplant to prevent host –rejection and the second for use in drug – eluting stent for eliminating restenosis of coronary arteries directly after angioplasty [9].

A wide array of clinically important secondary metabolites was produced from *Streptomyces* species [10,11]. Solid-state fermentation (SSF) represents the natural environment to which the bacteria and fungi can be cultivated and occur in the absence or near absence of free water. SSF is an excellent technique due to low production cost and easily applicable [12,13].

This work describes a report on the novel production of Rapamycin –analogue from a newly isolated *Streptomyces* sp. RHS5. The purification and structural elucidation of the therapeutic agent act as anti-*Candida* was elucidated. In addition, the economic production of the metabolite using wheat bran in solid state fermentation was approached, and finally the production was statistically optimized.

2 Materials and Methods

2.1 Microorganisms

Streptomyces sp. RHS5 used throughout the present work was isolated from agriculture soil and identified by 16S rDNA. *Streptomyces hygroscopicus* was kindly provided in a lyophilized form by Prof. Yasser R. Abdel-Fattah, Professor of Microbial Biotechnology, City of Scientific Research and Biotechnological Application. *Candida albicans* ATCC 1023, *Escherichia coli* ATCC 9739, *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538P were kindly provided by the Faculty of Pharmacy, Alexandria University.

2.2 Isolation of actinobacteria and screening for anti-*Candida* activity

10 g samples of soil and compost (10g) were pretreated by drying for 2 days at 60°C. Samples were transferred into 500ml flasks containing 100ml saline and shaked for 15 min. Serial dilutions were then prepared in saline solution and 1ml of each was spread onto agar plates of ISP1, ISP2, ISP4 media prepared as described by **Ayari et al. (2012)** [14], GYM *Streptomyces* medium [15]and starch nitrate [16] containing mycostatin (1ml / L) as antifungal agent. Plates were incubated for 4 to 7 days at 30°C. Colonies exhibiting powdery consistency and attached firmly to agar surface were picked and purified on the same medium using the spatial streaking method. The purified bacterial isolates were

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maintained on slants of GYM and starch nitrate with technical agar and kept at 4°C.

The **method [17] was adopted for screening anti-*candida* production. Agar discs** (9mm) were made using a sterile cork-borer from agar plates and transferred into 1.5ml Eppendorf tubes containing 0.5 ml methanol. After 30min shaking, methanol extracts were centrifuged for 15min at 7,000 rpm and the supernatants were then used for bioassay against *Candida albicans* ATCC 1023 using double agar diffusion plates. The base layer contained 15ml (glucose 5g/L, peptone 2g/L, agar 20g/L), and the top layer contained 0.1ml suspension of *Candida albicans* mixed with 5ml of sterile saline and 0.8% agar. Paper discs (5mm diameter) immersed in methanol extracts were placed on the surface of bioassay medium seeded with the test organism. A paper disc suspended in methanol served as a control. The plates were stored at 4°C for 12h, and then incubated for 2 days at 30°C. The zone of inhibition was measured in mm.

2.3 Identification of bacteria

The most potent isolate was identified phenotypically as **[18]**and phylogenetic analysis using 16S rDNA.

2.4 Enzyme profile and antimicrobial activity

Enzymatic activity was examined using starch, cellulose, casein, xylan and degradation of tween40 or 80 by adding 0.2% (w/v) of each substrate to nutrient agar medium and measuring inhibition zone. The antagonistic activity of *Streptomyces* strains were tested against *E.coli*, *Pseudomonas aeruginosa* (gram negative) and *Staphylococcus aureus* (gram positive) by agar well diffusion method [19].

2.5 Thin layer chromatography and Bioautography

The anti-*Candida* metabolite was extracted using methanol from mycelia after cultivation on GYM broth for 4 days at 30°C. Rapamycin was extracted by methanol from *Streptomyces hygroscopicus* mycelium after cultivation on ISP2 broth for 10 days at 30°C.The anti-*Candida* substance and rapamycin were subjected to thin layer chromatography using a solvent mixture of ethyl acetate: methanol: water (70:20:10) [20]. For bioautography, the silica gel plate was placed over Sabauraud's agar seeded with *C. albicans* and kept at 4°C for 12h to allow the anti-*Candida* substance and rapamycin to transfer and diffuse into agar . The silica gel plate was removed under sterilized condition and incubated for 2 days at 30°C. The inhibition zone was observed and the retention factor values (R_F) of the migrated anti-*candida* substance and compared with the R_F values of rapamycin produced by *Streptomyces hygroscopicus.* The R_F was measured from the origin line to the middle of inhibition zone.

2.6 Purification of anti-*Candida* substance

The methanol containing the active compound was concentrated by evaporation at 50- 60°C, yielding a yellow crude material which was dissolved in 10ml methanol and subjected to column chromatography using silica gel (3 × 15 cm) as a stationary phase. The column was developed with a solvent mixture of ethyl acetate: methanol (10:1 to 1:10) [21]. 24 fractions were collected and bioassayed for activity by disc diffusion method against *Candida albicans* ATCC1023 and the active fraction was evaporated in water bath at 50-60°C.

2.7 Characterization of the anti- *Candida* substance

the pure methanol extract was subjected to ultraviolet (UV) absorption (Perkin, Elmer spectrophotometer Lambda 4B) by its UV-visible spectrum, infrared (IR) absorption (BRUKER FTIR TENSOR 37) and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic analyses (JEOL JNM ECA 500MHz) in Central lab, Faculty of Science, Alexandria University.

2.8 Production of rap using solid state fermentation

10g of wheat bran were placed in 250ml flasks and moistened with mineral salt solution (g/L) $CaCO_3$, 1; NaCl, 0.2 $(NH4)_2SO_4$, 2.4 of pH 7.0 to reach a moisture level of 55%. After autoclaving, the cooled medium was inoculated with 2ml spore suspension containing 2.6×10^7CFU/ml *St.* sp. RHS5 and incubated at 30°C for six days. At the end of fermentation, the harvested biomass was treated with 30ml ethyl acetate [22] and shacked well for 30min.The whole content was soaked through sterile muslin cloth. The filtrate was pooled then centrifuged and the clear supernatant was used for inhibitory effect against *C. albicans* ATCC 1023.

2.9 Effect of initial moisture level

The effect of moisture level on anti-*Candida* production was evaluated by supplementing flasks containing 10g of wheat with different volumes of salt solutions to reach initial moisture content of 20, 30, 40, 50, 60 and 70%.

2.10 Optimization of Rap production using Plackett-Burman

Statistical optimization is an economic, efficient and accurate approach that allows maximization of the microbial secondary metabolites yield and estimates the relevance of the strain for its use in an industrial scale production. Plackett- Burman (PB) design is a powerful and efficient mathematical model to determine and screen out the effect of parameters [23]. This design is very practical, especially when the investigator is faced with a large number of factors and is unsure which settings are likely to be close to optimum responses. PB was used to identify the major fermentation parameters that affect anti- *Candida* production. The experimental design was applied with 7 different variables **(Table 1**) and the design matrix was developed using Statistica software (trial version 6.0, StatSoft, USA). All experiments were carried out in triplicate and the averages of the results were taken as response (**Table 2)**. For each variable, a high (+) and a low (-) level was tested. All trials were performed in triplicate and the average of production observation results were treated as shown in the equation:

$$\text{Main effect} = (\sum R(H) - \sum R(L)/N$$

Where R (H) = all responses when component was at high levels, R(L) = all responses when component was in low levels, N= total number of trials divided by 2.

3. Results and Discussion

Among the isolated actinobacteria, R5 depicted the highest inhibition zone (20 mm) against *Candida albicans*. Morphological characterization (**Fig. 1),** and physiological properties (**Table 3)** revealed the assignment of R5 to Genus *Streptomyces*. This was confirmed by 16S r DNA sequence analysis showing 99% similarity to *Streptomyces rochei* strain AC23 and was thus designated as *Streptomyces* sp. RHS5. The

sequence was deposited in Genbank under accession number JX028274 (**Fig. 2)**. Strain RHS5 showed antagonistic activity against *Staphylococcus aureus* and *E.coli* forming inhibition zones of 12.5 and 11mm, respectively.

3.1 Bioautography

The rate flow (R_F) of anti-*Candida* agent produced by the *Streptomyces* RHS5 strain was compared to R_f of rapamycin produced by *Streptomyces hygroscopicus* using TLC. As **Table 4**, data depicted that the compound produced by *Streptomyces* sp.RHS5 was a rapamycin analogue

3.2 Chemical identification of the active metabolite

The active metabolite produced four characteristic Utraviolet(UV) visible spectrum peaks at 280, 425and 473nm with maximum absorbance at 239nm (**Table 5**) indicating the presence of a carbon-carbon double bonds and hence confirming the polyene nature of the anti-*Candida* metabolite. The fourier-transform infrared spectroscopy(FTIR)- spectrum of the pure compound (**Fig. 3)** evidenced a diagnostic peak at 3431.44cm^{-1}, indicative to the presence of OH group, the peak at 2844.49 was assigned to alkane (CH, sp_3), the peak at 1645.30$cm^{-1}$was assigned to alkenes (C=C) group or carbonyl (C=O) group of the amide functional group. The peak appearing at 1109.86cm^{-1} was assigned to (C-O ether) group of the ketone functional group. The data of FTIR- spectrum coincides with the functional groups of rapamycin chemical structure except in the absence of carboxylic (COOH) group and ester (COOR) alkynes. These results are similar to those obtained and identified active metabolite as 7-demethoxy rapamycin [9].

The^{1}H-nuclear magnetic resonance (NMR) spectrum of the active metabolite (**Fig. 4**) was compared with that of rapamycin [24]. The result suggests that the number of olefinic proton (27) from 5.29 to 6.39 of active metabolite was more than the number of olefinic proton of rapamycin (14). It also showed that the number of aliphatic protons, from 0.95 to 4.17, of active metabolite (52) is less than number of aliphatic protons of rapamycin (65).

3.3 Production of RAP using wheat bran as substrate for solid state fermentation.

To reduce the production cost, wheat bran was assessed as substrate for rapamycin production in solid-state fermentation (**Fig. 5**). Wheat bran is readily available in Egypt in large quantity as a low-cost by-product [25]. wheat bran proved to be a potent solid substrate to produce antibiotics by *Streptomyces* sp. AS4 and used for rap production by *Streptomyces hygroscopicus* subsp. *ossamyceticus* (strain D10) [22]. Whereas, soybean and corn meal were used by *Streptomyces hygroscopicus* BS-112 [26].

3.4 Optimizing the moisture level.

IN SSF process, moisture content plays a vital role. Data of this work reports that an initial moisture level of 50% supported the production of *St.* sp.RHS5 anti-*Candida* substance with an inhibition zone diameter of 10mm.At lower moisture content (20- 40%), the available oxygen is sufficient but the water content was low to support metabolic activity and elimination of the heat generation [27]. Increase of the moisture level from 60 to 70% lead to decrease of available oxygen as result of replacement of air present by water. The initial moisture level was 70% for neomycin production from apple pomace by *Streptomyces fradiae* NCIM 2418**[28]**, whereas, the optimum moisture content as 65% for the production of oxytetracycline by *Streptomyces speibonae* OXS1 from cocoyam peels **[29]**. Moreover, neomycin production by *Streptomyces marinensis* using wheat bran at moisture content of 80% **[30].**

3.5 Optimization process using Plackett-Burman

The Plackett- Burman experimental design matrix for 9 trials at two levels of concentration for each variable with two center points along with the respective experimental response (zone of inhibition in mm) are given in **Table 2.** Largest zone of inhibition (mm) is directly correlated with higher antagonistic activity. The values of inhibition zones diameter for different treatments showed a variation from zero to 13.5 mm, suggesting that the studied variables had a significant effect on the inhibition zone and thus on production of bioactive compound.

Main effects allow the determination of the effect of each constituent. A large contrast mean, either positive or negative, indicates that a factor has a large impact on titre; while a mean close to zero indicates that a factor has little or no effect. When the sign of the effect of the tested variable is positive, the influence of the variable on production process is greater at a high level, but the negative sign indicates that the effect of the variable is greater at a low level. Main effects of the examined variables were calculated and represented graphically in **Fig. 6.**

As clearly shown production of rapamycin analogue by *St.* sp. RHS5 was positively affected by glucose, salt solution, yeast extract, KH_2PO_4, and incubation period, while negatively affected by wheat bran and inoculum size within the tested range. Positive effect explains that if a higher concentration was used, a better response was achieved, while a negative effect means lower concentrations are favored for better results. So, rapamycin analogue production was mainly dependent on KH_2PO_4 concentration. Under our experimental condition, increasing the KH_2PO_4 concentration in the culture medium increased rapamycin analogue while the glucose and soybean powder had a positive effect, while KH_2PO_4 had a negative effect on the production of the antifungal active substance by *Streptomyces hygroscopicus* BS-112 [26]. On the other hand, rapamycin production was increased by optimizing the concentrations of mannose, soybean meal, and l-lysine [31].

Table 6 shows the statistical parameters obtained after the Design-Expert analysis. Analysis of variance (ANOVA) was performed in order to find the effect and contribution of each variable. The *P* values were used as a tool to determine the significance of each of the coefficients. The smaller the magnitude of *P*, the more significant is the corresponding coefficient. Values of p less than ≤ 0.05 indicate model terms that are significant. The coefficient and the corresponding p values suggest that, among the input variables, only potassium dihydrogen phosphate was significant model term. Some investigators employed confidence levels greater than 70 % as significant effect levels [32] where the confidence levels greater than 85 % are acceptable [33]and other investigators find that, confidence levels greater than 90%are acceptable [34].

Table 1 Factors examined as independent variables affecting rapamycin production by *Streptomyces* sp. RHS5 and their levels in the Placket-Burman experimental design

Factor	Symbol	Low level (-)	Basal (0)	High level (+)
Wheat bran (g)	WB	4	6	8
Salt solution component (g/l)	SS	(0.5+0.1+1.2)	(1+0.2+2.4)	(2+0.4+4.8)
Glucose (g)	G	0.04	0	0.08
Yeast extract (g)	YE	0.04	0	0.08
KH_2PO_4 (g)	KH	0.5	0	1
Inoculum size (ml)	IZ	1	2	3
Incubation period (day)	IP	4	6	8

Table 2 The Placket-Burman experimental design (in coded levels) with zone of inhibition (mm) as response

Factor	Independent variable							Response
	WB	SS	G	YE	KH_2PO_4	IZ	IP	Inhibition zone (mm)
1	+1	- 1	- 1	+1	-1	+1	+ 1	0
2	+1	+1	- 1	- 1	+1	-1	+1	11
3	+1	+1	+1	- 1	- 1	+1	-1	0
4	-1	+1	+1	+ 1	-1	- 1	+1	7
5	+1	- 1	+1	+1	+1	- 1	-1	8
6	-1	+1	-1	+1	+1	+ 1	- 1	10
7	-1	-1	+1	- 1	+1	+1	+1	9
8	-1	-1	-1	-1	-1	-1	-1	0
9	0	0	0	0	0	0	0	13.5

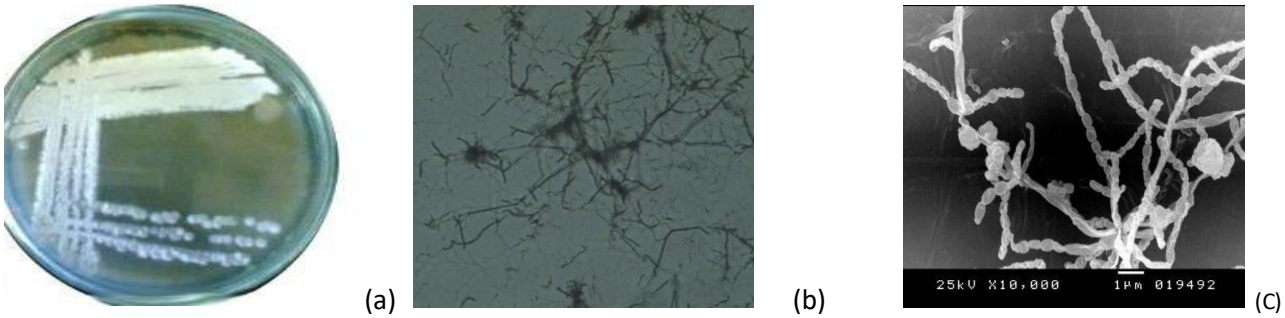

Fig. 1 (a): Morphology of the bacterial strain on GYM after 4 days (b): Spore chain morphology:rectus-flexibilis (40x) in R5 at light microscope (c):Scanning electron micrograph (10,000xs) showing straight spore of R5 growing on GYM for 4 days at 30°c

Table 3. Morphological and physiological characteristics of the two isolates

Name of the test	**Bacterial isolate (R5)**
Gram stain	+
Pigmentation	Brown
Growth	Good
Cell wall diaminopimelic acid (DAP)	present
Catalse	+
Oxidase	-
Citrate utilization	+
Urea hydrolysis	+
Tryptophan hydrolysis	-
Gelatin hydrolysis	+
Starch hydrolysis	+
Casein hydrolysis	+
Cellulose hydrolysis	+
Tween 80 hydrolysis	+
Xylan hydrolysis	+
Tween 40 hydrolysis	+

Table 4 The rate flow of the active metabolite using TLC

	The active metabolite	***Rate flow(cm)***
Streptomyces RHS5.sp		0.71
Streptomyces hygroscopicus		0.77

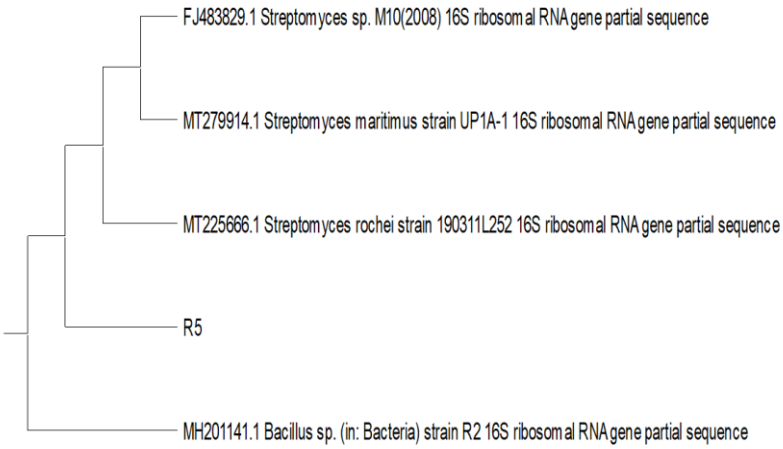

Fig. 2 Phylogentic tree of isolate R5. 16s rDNA- based dendogram showing the phylogenetic position of isolate R5 among representative of related bacterial species

Table 5 UV absorbance of *Streptomyces* sp. RHS5 metabolite

Bacterial isolate	**Max (nm)**	**UV absorbance shoulder**
Streptomyces sp. RHS5	280,425,473	239

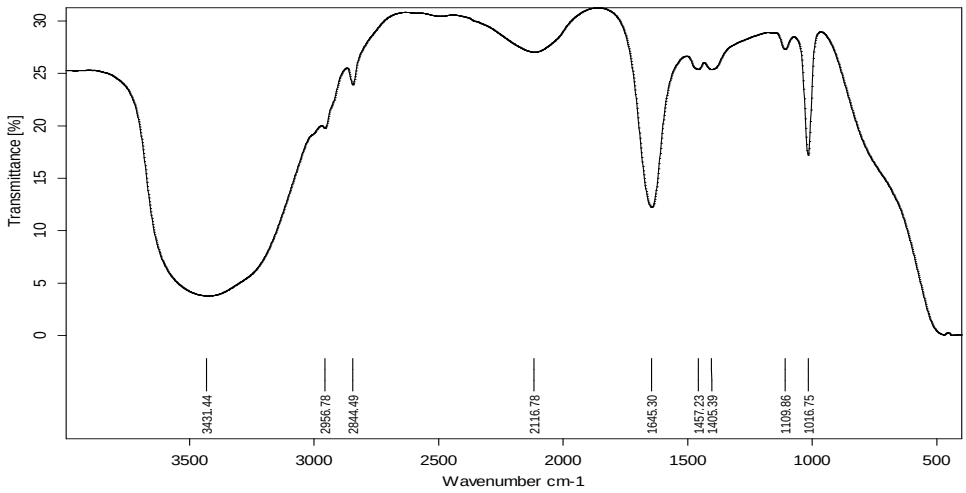

Fig.3 FTIR-spectrum of anti-*Candida* compound produced by *Streptomyces* sp.RHS5

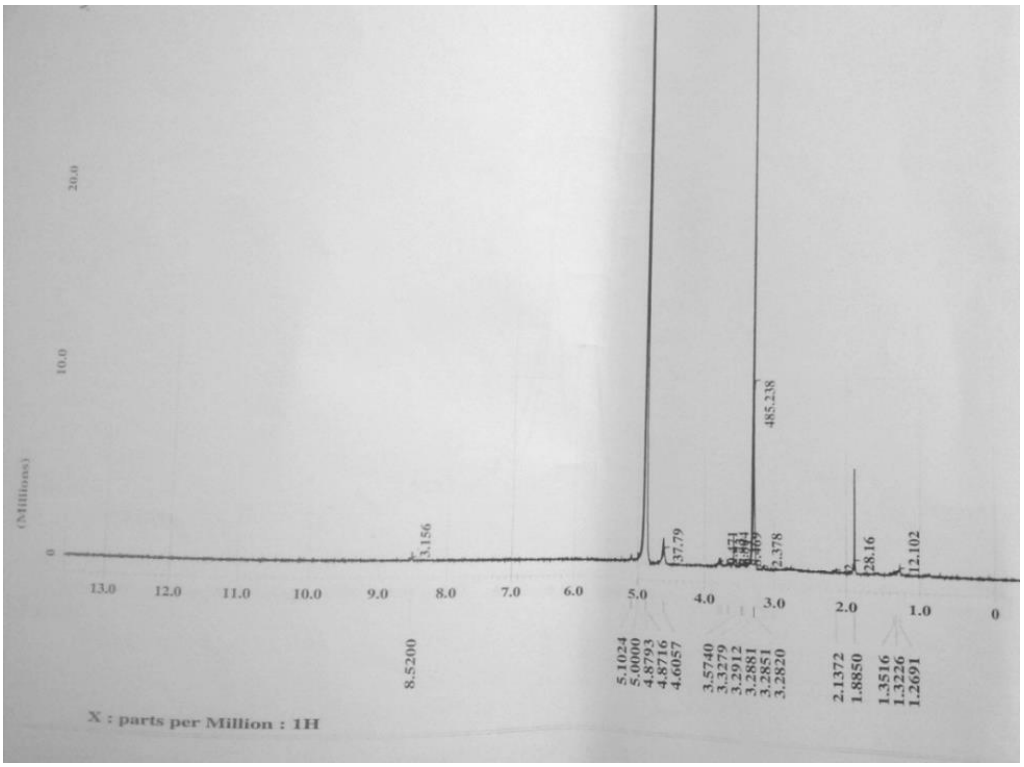

Fig. 4 ^{1}H-NMRspectrum of *Streptomyces* sp. RHS5 anti-*Candida* compound.

Fig.5 Growth of *Streptomyces* sp. RHS5 on wheat bran

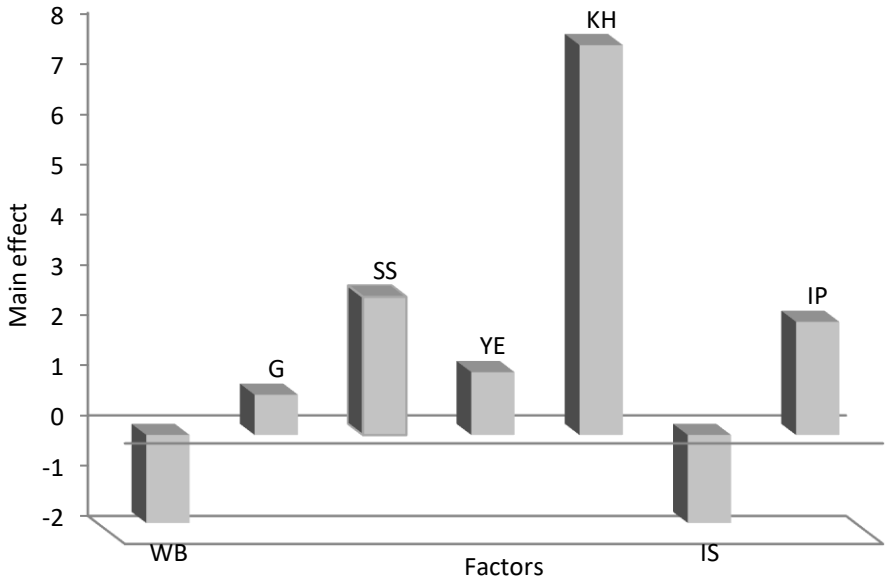

Fig. 6 Elucidation of cultivation factors affecting rapamycin analogue produced by *Streptomyces* sp. RHS5

3.6 Validation of the model

To validate the obtained data and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates. The optimized medium was thus composed of (g/ flask) wheat bran, 4; glucose, 0.08; yeast extract, 0.08; KH_2PO_4,1; and salt solution (g/l) $CaCO_3$, 2; NaCl, 0.4; $(NH_4)_2SO_4$, 4.8 with inoculum size (1ml) for 8 days. The anti-optimized medium was the opposite concentration of the optimized and its growth medium formula was (g / flask) wheat bran, 8; glucose, 0. 04; yeast extract, 0. 04; KH_2PO_4, 0.5 and salt solution (g/L) $CaCO_3$, 0.5; NaCl, 0.1; $(NH_4)_2SO_4$, 1.2 with 3ml inoculum size and incubation for 4 days.

Table 6. Statistical analysis of the Plackett-Burman experimental result

Factor	Main effect	T-value	P-value	Significance
Wheat bran	-1. 75	0. 47735571	0. 64437174	35%
Glucose	0. 8	0. 19913741	0. 84571835	15%
Salt solution component	2. 75	0. 77094466	0. 46156268	54%
Yeast extract	1. 25	0. 33364383	0. 74281625	26%
KH_2PO_4	7. 75	3. 74694739	0. 01420335	98%
Inoculum size	-1. 75	0. 47735571	0. 64437174	35%
Incubation period	2. 25	0. 63313531	0. 55025601	45%

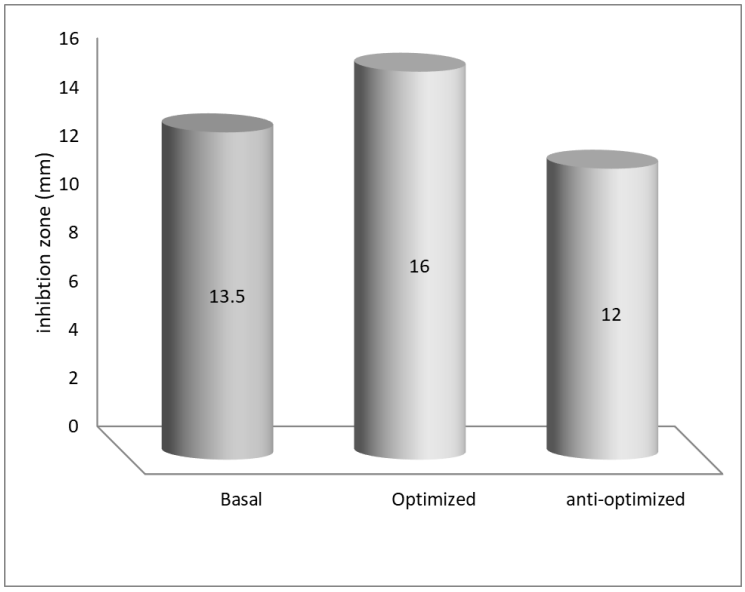

Fig. 7 Verification experiment of the applied Plackett-Burman statistical design by comparing the rapamycin analogue produced by *Streptomyces* sp. RHS5 growing on the resulting optimized medium, the basal medium and the antioptimized medium

The data were examined and compared to the basal and anti-optimized medium. As we notice from **(Fig.7)**, the verification experiment indicates that the zones of inhibition of rapamycin analogue before and after optimization were 13.5 and 16mm, respectively. Thus, a significant increase (1.18-fold) in the antagonistic activity was achieved by Plackett-Burman optimization.

4.Conclusion

The present study provides information on the production of rapamycin analogue, an antifungal antibiotic effective against *Candida albicans*, from a newly local isolated species of the genus *Streptomyces*. To the best of our knowledge, this is the first report on the production of this bioactive compound from another species rather than *Streptomyces hygroscopicus.* Thenovel *Streptomyces*. sp. RHS5 is a multifunctional organism producing antibacterial agents, enzymes in addition to rapamycin analogue. The structure of the active compound was elucidated after extraction and purification through the application of IR, UV and ^{1}H-NMR analysis. Moreover, the production of the desired compound was economically investigated by applying the solid-state fermentation using wheat bran as a substrate. Optimization of culture condition was approached by a statistical design named Plackett- Burman, and the data indicated the validation of the process. The information provided opens a new avenue to produce novel pharmaceutical compounds from local bacterial species.

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