Volume 10, Issue 1, 2020, 4811 - 4820 ISSN 2069-5837
Biointerface Research in Applied Chemistry
www.BiointerfaceResearch.com
https://doi.org/10.33263/BRIAC101.811820
Original Research Article Open Access Journal
Received: 30.09.2019 / Revised: 16.12.2019 / Accepted: 20.12.2019 / Published on-line: 28.12.2019

# *Thermoflavimicrobium dichotomicum* as a novel thermoalkaliphile for production of environmental and industrial enzymes

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## ABSTRACT

Thermoalkaliphilic actinomycetes enzymes have many important applications in many industrial, biotechnological and environmental aspects. So, the current study aimed to obtain the thermoalkali-enzymes producing actinomycetes. A novel thermoalkaliphilic actinomycete strain was isolated from Egyptian Siwa oasis and identified according to its morphological, physiological and biochemical characters as *Thermoflavimicrobium dichotomicum*. And then confirmed by phylogenetic analysis and the partial sequence was deposited in GenBank under accession number of KR011193 and name of *Thermoflavimicrobium dichotomicum* HwSw11. It could produce amylase, cellulase, lipase, pectinase and proteinase enzymes. Also, this strain exhibited anti-bacterial activities against *P. aeruginosa* and *E. coli* with inhibition zones of 14 and 20 mm, respectively. Consequently, it has antifungal activity against *A. niger*, *A. flavus* and *Penicillium notatum* with inhibition zones of 17, 14 and 14 mm, respectively. For that, it may be concluded that *Thermoflavimicrobium dichotomicum* HwSw11 as a novel thermoalkaliphile has a wide range of biological activities against a broad spectrum of pathogenic bacteria and fungi, in addition to produce many enzymes (amylase, cellulase, lipase, pectinase and proteinase). So, this isolate could be applied as manufactory for many industrial, biotechnological and environmental sectors.

**Keywords:** *Antimicrobial activity; Enzymes activity; Thermoalkaliphilic actinomycetes; Thermoflavimicrobium dichotomicum.*

## 1. INTRODUCTION

New biotechnology deals to search about new isolates that have ability to grow and work at harsh conditions like extreme temperatures and pH values for using it in industrial, biotechnological and environmental applications [1-3]. Also, it goes up for solving problems by producing of producing non-traditional materials as antimicrobial agents [4-6], nanobio-synthesized metals like silver, copper and zinc [7,8] and biotechnological agents as bioactive materials [9-12]. Extremophile microorganisms have various adaptation mechanisms enabling them to thrive and produce secondary metabolites under harsh conditions [13-15]. Extremophiles are an organisms that grow under harsh environmental conditions such as temperatures (≥ 50 ℃) or (<10 ℃), alkaline (≥ 8.5) or acidic (≤ 5) pH values, high salinity and high pressure [16-18]. Extremophiles isolates from different habitats include soil, compost, manure [19], geothermal areas [20], sewage sludge or municipal solid waste [21], oil wells [22] and thermally treated foods [23].

Egypt has many harsh environments that might be a good source for isolation of extreme actinomycetes. Swia oasis is one of these places [24], it extreme heat in summer, warm wintertime and very cold at night [25, 26], for that it is a significant site for isolation new isolates. Thermoalkaliphilic actinomycetes have especially potential industrial applications because it has enzymes stable at a high pH and temperatures values [27]. And it produces resistant endospores and survive in harsh environments for a long period, up to 9000 years [28,29]. The adaptation in *actinomycetes* to survive at high temperature is due to the presence of membrane lipids, which contains a more saturated and straight chain of fatty acids [30,31]. Extremophile possesses novel and highly functional activities, it is a powerful source of many useful products such as hydrolytic extracellular enzymes, Protein, antibiotics, hormones… etc [32-34].

Thermostable enzymes could catalyze important chemical reactions in several industrial and research fields including food, agricultural, pharmaceutical, cosmetic, as well as several other vital economic aspects [35]. Extremophilic actinomyces species could be a source of thermostable enzymes, which are not only heat resistant, but also stable under highly alkaline conditions [36,37]. It has been reported before that *Thermoflavimicrobium dichotomicum* genus could produce several vital thermostable enzymes such as lipase and proteinase [38], these facts drove us to explore the ability of the isolated novel *Thermoflavimicrobium dichotomicum* to produce lipase, amylase, cellulase, pectinase and proteinase enzymes. The ability of any species to produce antimicrobial secondary metabolites exaggerates the opportunity to be used in industrial sectors. So, the current study is concerning the isolation of *Thermoflavimicrobium dichotomicum* from Siwa oasis in Egyptian desert as a useful manufactory for biotechnological and environmental applications.

## 2. MATERIALS AND METHODS

### 2.1. Sample collection and preparation

A total of 12 sandy soil samples (approximately 500 g of each) were collected from seven different sites at Siwa oasis, Marsa Matrouh governorate, Egypt. Each site has been taken special codes and the texture of these samples was determined. The samples were collected in clean plastic bags from layers of 15 to 40 cm depth and air dried for 3 days at 55°C [39, 40]. The pH of soil extract was measured using digital portable pH meter Adwa® AD11 and the soil salinity was measured by digital portable TDS meter Adwa® AD201. The samples were mixed with $CaCO_3$ (1g/ 100g soil) for 24 hrs at 28 °C before plating to increase the numbers of actinomycetes.

### 2.2. Isolation and purification of thermoalkaliphilic actinomycetes.

Isolation and enumeration of actinomycetes were performed by soil dilution plate technique using different media. Culture media include Czapek's yeast casamino acid (CYC) agar medium [41], Nutrient agar [42], Plate count agar medium [43], Modified Bennett's agar [44], Inorganic salts-starch agar medium (ISP-4) [45] and Starch-nitrate medium [46]. All media were adjusted to pH 8 and all then inoculated and incubated at 55 ºC for 1, 2 and 3 weeks. Selected colonies (rough, chalky) of actinomycetes were selected and transferred to respective agar plates and incubated for 7 days. Plates containing pure cultures were stored until further examination.

### 2.3. Selection of the most promising isolate.

The most potent isolate was determined depending on the highest tolerance to temperature and alkalinity. All actinomycetes isolates were cultured onto respective agar medium and incubated for 7 days at different temperatures (40, 45, 50, 55, 60 and 65°C) [47] and with different pH values (6, 7, 8, 9, 10, and 11) [48].

### 2.4. Screening for enzymes assay of the isolated actinmomycetes.

Hydrolysis enzymes like amylase, protease, lipase, cellulose and pectinase were assayed in this experiment. Behavior of the actinomycetes isolates toward different substrates of the selective enzymes was noted on agar medium. Pure isolates with maximum growth range (on agar plates) were cultured on basal mineral salts agar medium with addition of glucose for control, starch for amylase [49], casein for protease [50], tributyrin for lipase [51], carboxy methyl cellulose for cellulose [52] and pectin for pectinase [53].

### 2.5. Identification of the most potent actinomycete isolate.

HwSw11 isolate as the most active one in production of selective enzymes was characterized and identified using morphological, physiological, biochemical methods [42] and molecular techniques [54].

In case of morphological and culture characteristics, HwSw11 isolate were cultivated and observed on different ISP (1, 2, 3, 4, 5, 6 and 7) agar media [42] and non ISP media such as, CYC agar medium [41], Nutrient agar [42] and Starch-nitrate medium [46]. All media adjusted to pH 8 and incubated for 4-7 days at 55°C. Aerial spore mass colour, substrate mycelium colour and the colour of any diffusible pigments were recorded using the National Bureau of Standards (NBS) Colour Name Charts [55]. The morphology of spore chains and sporophores was observed by direct light microscope (Optika, Italy) using cover slip technique [56], also spore morphology was examined by a scanning electron microscope [57] (JEOL Technics Ltd, Japan) at the regional center for mycology and biotechnology, Al-Azhar University, Cairo. In case of Chemotaxonomy characteristics, the determination of the isomeric form of diaminopimelic acid was done as described by Hasegawa et al. [58]. The analysis of the sugar composition of cell wall was carried out using the method described by Lechevalier and Lechevalier [59], using sugars of arabinose, galactose, glucose, ribose, mannose and xylose in cell hydrolysates.

For Physiological and biochemical characteristics, Isolate HwSw11 was investigated on Yeast – malt extract agar medium (ISP-2) at 55 ºC and pH adjusted to 8 to utilize different carbon and nitrogen sources, NaCl concentrations suppressed the growth, Melanin production was observed on the following media Peptone yeast extract-iron agar medium (ISP 6), Tyrosine agar medium (ISP 7), and Tryptone - yeast extract broth medium (ISP-1) [42, 60]. On the other hand, Biochemical tests including catalase production, gelatin liquefaction, H2S production, Nitrate reduction [61], antibiotic sensitivity, Melanin production [42], were also determined.

In the case of sensitivity to antibiotics, 14 different antibiotics were screened using disc diffusion method to determine the sensitivity of the target isolate to these antibiotics. Disks impregnated with penicillin, chloramphenicol, erythromycin, bacitracin, clindamycin, rifampicin, vancomycin, oxicillin, ciprofloxacin, ofloxacin, nalidixic acid, lincomycin, norfloxacin, and tetracycline were placed on the inoculated agar plates [40]. The zones of growth inhibition were measured and the sensitivity was calculated [62].

The characteristics of the HwSw11 isolate were compared with those of reference strains (*Thermoactinomyces vulgaris, Thermoflavimicrobium dichotomicum, Laceyella sacchari* and *Laceyella putida*) obtained from Bergey's manual of systematic bacteriology, 2nd ed, volume three, the Firmicutes [38].

Phylogenetic identification was applied to confirm characterization and identification of the target isolate. DNA extraction was performed by lysozyme-sodium dodecyl sulfate method [63]. A partial 16S rRNA gene was amplified using primers; RW01 (5′-AACTGGAGGAAGGTGGGGAT-3′) and DG74 (5′AGGAGGTGATCCAACCGCA-3′) [64]. PCR product was purified using QIA quick PCR purification kit (Qiagen). Sequence analyses were carried out by Sanger Sequencing Technology on Applied Bio-systems automated DNA sequencer, model ABI 3730XL DNA Analyzer (Applied Bio-systems, USA; service provided by Macrogen Inc., South Korea). The sequence analyses and alignments were performed by NCBI-BLAST programs [65, 66]. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software [67]. The phylogenetic tree was displayed using the TREEVIEW program [68].

### 2.6. Antimicrobial activity evaluation of the secondary metabolites of HwSw11 strain.

Antimicrobial activity of the cell free cultivation medium was determined by Agar well-diffusion method [69]. Pathogens used for testing antimicrobial activity were the reference strains;

*Bacillus cereus (*ATCC-12228), *Staphylococcus aureus* (ATCC-6538), *Pseudomonas aeruginosa* (ATCC-9027), *Escherichia coli* (ATCC-7839), *Candida albicans* (ATCC-10231), *Aspergillus niger* (NRRL-348), *Aspergillus flavus* (ATCC-16883), *Fusarium moniliforme* and *Penicillium* sp. *Thermoflavimicrobium dichotomicum* HwSw11 strain was grown in Starch-nitrate broth medium for 3 days with pH 8 at 50 ºC and then centrifuged and the supernatant was applied as antimicrobial agent. All experiments were performed in triplicate, the mean and standard error values were calculated [70, 71].

**2.7. Optimization and examination of beneficial enzymes production.**

Five beneficial enzymes were assessed in this study. Amylase enzyme was assessed using soluble starch as a method recorded by Kurup et al. [49]**.** Protease activity was determined using L-tyrosine as a standard. Five milliliters of 0.65% (w/v) casein in 50 mM potassium phosphate buffer, pH 8.5 is added to 1 ml enzyme solution and the assay mixture was incubated for 10 min at 55 ºC in the water bath. After the incubation was done, 5 ml of 110 mM trichloroacetic acid reagent (TCA) was added to enzyme-substrate solution to terminate the reaction. The mixture was put in ice bath for 10 min and centrifuged at 13500 rpm, at room temperature for 10 min then the supernatant was collected. The color development reaction was done by adding 2 ml of supernatant to 5 ml of 500 mM sodium carbonate solution followed by the addition of 1 ml of Folin Ciocalteu's phenol reagent into a tube and mixed by swirling. The reference tube had the same composition except for the enzyme solution. The changing of the activity was recorded by spectrophotometer in absorbance at 660 nm after 30 min. The enzyme activity was given as unit (U) and one unit is that hydrolyze casein to produce color equivalent to 1.0 µmole (181.0 µg) of tyrosine per min under the defined assay conditions. Lipase was tested using 1% tri butyrin as detailed by Hussein et al. [51]. Also, cellulase was assayed using carboxy methyl cellulose as mentioned by Hasanin et al. [52]. In case of pectinase, the enzyme activity was determined using pectin as a substrate. The assay mixture consists of 0.80 ml of the substrate solution (1.0% w/v citric pectin in 0.1 M citric buffer pH 5.0) and 0.20 ml of enzyme solution. The reaction medium was incubated at 50°C for 20 min and terminated by the addition of 1.5 ml of 3, 5-dinitrosalicylic acid reagent. The absorbance of the reaction medium was taken at 540 nm. One unit of pectinase activity (U) was defined as 1 µmol reducing sugar released per minute using galacturonic acid as standard.

The tested enzymes were optimized for production at deferent values of pH and degrees of temperature. The activity was recorded as an average of three replicates.

## 3. RESULTS AND DISCUSSION

**3.1. Samples collection and soil analysis.**

Recently, several studies have been made to obtain new actinomycetes from harsh habitats like extreme temperature, pH, heavy metal and salinity for production of different bioactive agents as enzymes, anticancer and antibiotic compounds [13, 14]. One of the most essential steps for isolation of the extremophiles is the samples collection. So, this study was concerned about collection of poly extremophiles' samples. High alkalinity, high TDS and geothermal nature of Siwa oasis will be a precise choice for isolation of thermoalkaliphilic actinomycetes [72]**.** Twelve sandy soil samples were collected from seven different locations at Siwa oasis. Physicochemical analysis of the collected samples clearly revealed variation in pH ranging from 8.1 to 9.5 that was chosen to be used for adjustment of screening media (**Table 1**).

**Table 1.** Physicochemical properties of the collected soil samples from Siwa oasis

| Code | Physicochemical analysis | |
|---|---|---|
| | TDS (ppm) | pH |
| **SW1** | 1940 | 8.6 |
| **SW2** | 7330 | 8.1 |
| **SW3** | 11100 | 8.3 |
| **SW4** | 3300 | 9.0 |
| **SW5** | 3680 | 8.9 |
| **SW6** | 200 | 8.7 |
| **SW7** | 300 | 8.8 |
| **SW8** | 1550 | 8.1 |
| **SW9** | 770 | 8.4 |
| **SW10** | 1600 | 8.1 |
| **SW11** | 9800 | 8.3 |
| **SW12** | 8350 | 8.6 |

Total dissolved solids (TDS) values were ranged from 200 to 11100 ppm; the lowest concentration was recorded in Sw6 sample (200 ppm), while the highest concentration recorded in Sw3 sample (11100 ppm).

**3.2. Isolation and Selection of the most promising thermoalkaliphilic actinomycete isolate.**

Different culture media were used for isolation of thermoalkaliphilic actinomycetes. A total of 20 actinomycete isolates (coded from HwSw1 to HwSw20) were isolated from 12 sand soil samples on different agar media. The little number of actinomycete isolates may be due to geothermal environment and a little microorganism able to grow in extreme conditions [41, 73]**.**

These isolates were screened for their ability to resist extreme temperatures and high pH values. Among them, actinomycete HwSw11 exhibits a good growth at high temperature and high alkalinity; it can grow at a range of pH (7 to 12), with an optimum pH 9. Also, it can grow in the temperature range between 40 and 55 ºC, with an optimum temperature at 50ºC. These may be due to the environment of this isolate at Siwa oasis. For that, it may be based on this isolate for the production of biologically active agents. Also, Barakat et al. [15] obtained some isolates from red sea, Egypt as haloalkaliphile depended on the site of isolation.

The obtained isolates were screened for the ability to produce some industrial and environmental enzymes like amylase, cellulase, lipase, pectinase and proteinase on solid agar medium (**Fig. 1**). From all tested isolates, isolate HwSw11 showed great activities against all of the substrates under this study. So, it selected as the most potent extremophilic isolate in this study and chosen for the identification studies as a new isolate.

**3.3. Identification of the isolate HwSw11.**

Microscopic examination of isolate HwSw11 revealed that; aerial mycelium was abundant and distinctive on nutrient agar media after 24 h at 55°C under simple microscope (X10) and the

colonies were observed yellow to orange with entire margins (Fig. 2). Spore chains also observed under light microscopy, it showed a dichotomously branched mycelium, sporophores with single spores and the aerial mycelium appeared to be fragmented with aging of culture. In general, the spore's chains appeared to be categorized as Rectus-Flexibilis (RF), and Monoverticillus (MV) (Fig. 3).

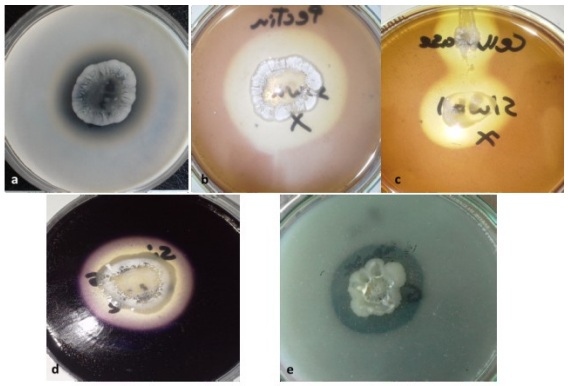

**Figure 1.** Screening for enzymes assay production by isolated actinomycetes like proteinase (a), pectinase (b) cellulose (c), amylase (d) and lipase (e) on solid agar medium.

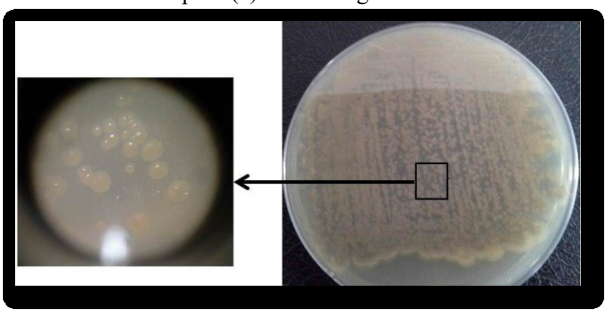

**Figure 2**. Colonies of HwSw11 observed under simple microscope 10 x on nutrient agar media after 24 h at 55ºC.

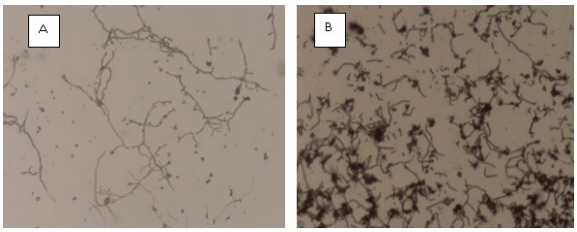


**Figure 3**. A, Autolysis of mycelium; B, Hyphae structure observed under light microscope 400x.

Under scanning electron microscopy (SEM), it was found that the isolate HwSw11 produced rectus flexible spore chains (Fig. 4A) and the spores appeared to be spheroidal, 0.5–1.5 µm in diameter, with a ridged surface that gives an angular appearance (Fig. 4). Also, the cell wall peptidoglycan hydrolysis showed that, it contains meso-diaminopimelic acid and no characteristic sugars pattern.

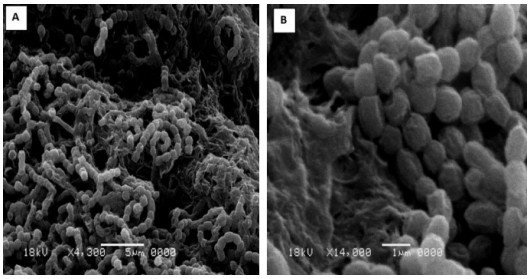


**Figure 4**. SEM of isolate HwSw11 spores grown on ISP2 agar, 96 h, **A,** 4300x and B, 14000x.

In the case of Culture characteristics, isolate HwSw11 was grown well on all media except oat meal agar (ISP 3) showed a weak growth as shown in **Table (2)**. Isolate HwSw11 forming a yellow colour substrate mycelium on Yeast – malt extract agar (ISP 2), Inorganic-trace salt- starch agar (ISP 4), Tyrosine agar (ISP 7) and Nutrient agar.

For physiological and biochemical characteristics, isolate HwSw11 can utilize a wide range of sugars added to ISP 2 medium as a sole carbon source, D-Glucose and Sucrose appeared to be the best carbon source stimulating the growth. This isolate investigated on different nitrogen sources and exhibited good growth with beef extract as a sole nitrogen source, moderate growth with peptone and weak growth with urea and ammonia. NaCl concentrations suppressed the growth starting from 1 % and above. Also, it has ability to produce catalase and liquefaction of gelatin in media. On the other hand, it hasn't ability to reduce nitrate and can't produce $H_2S$ and melanin pigment on investigated media.

**Table 2.** Cultural properties of isolate HwSw11.

| Nutrient medium | Growth | Color of the aerial mycelium | Color of the substrate mycelium | Soluble pigment |
|---|---|---|---|---|
| Tryptone yeast extract agar (ISP 1) | Moderate | Cream-white | Light grey | Pale yellow |
| Yeast – malt extract agar (ISP 2) | Good | Light grey | Yellow | None |
| Oat meal agar (ISP 3) | Weak | Hygroscopic | Cream-white | None |
| Inorganic-trace salt-starch agar (ISP 4) | Good | White | Pale yellow | None |
| Glycerol asparagine agar (ISP 5) | Moderate | Hygroscopic | Beige | None |
| Peptone yeast extract iron agar (ISP 6) | Good | Dark-brown | Brown | Yellow |
| Tyrosine agar (ISP 7) | Good | White | Deep-yellow | Pale-yellow |
| Starch-nitrate agar | Good | White | Beige | Dark-beige |
| Nutrient agar | Good | Cream-white | Yellow to orange | None |
| CYC agar | Good | Orange | Brown | None |

**Table 3.** Antibiotic sensitivity of isolate Sw11.

| Antibiotics Name | Conc. µg/ml | Zone diameter interpretative standards R | I | S | Isolate sterile zone (mm) | Isolate resistance and susceptibility |
|---|---|---|---|---|---|---|
| **Bacitracin** | **10** | 12 | 12-14 | 14 | 20 | S |
| **Clindamycin** | **2** | 14 | 15-20 | 21 | 0 | R |
| **Penicillin** | **10** | 27 | 28-29 | 30 | 20 | R |
| **Chloramphenicol** | **30** | 15 | 16-17 | 18 | 17 | I |
| **Erythromycin** | **15** | 13 | 14-22 | 23 | 0 | R |
| **Rifampicin** | **30** | 20 | 21-25 | 26 | 25 | I |
| **Vancomycin** | **30** | 10 | 11-14 | 15 | 25 | S |
| **Oxicillin** | **1** | 10 | 11-12 | 13 | 0 | R |
| **Ciprofloxacin** | **5** | 13 | 14-20 | 21 | 20 | I |
| **Ofloxacin** | **5** | 12 | 13-15 | 16 | 20 | S |
| **Nalidixic acid** | **30** | 13 | 14-18 | 19 | 0 | R |
| **Lincomycin** | **2** | 14 | 15-19 | 21 | 0 | R |
| **Norfloxacin** | **10** | 12 | 13-16 | 17 | 15 | I |
| **Tetracycline** | **30** | 14 | 15-18 | 19 | 23 | S |

In another case, this isolate was sensitive to some tested antibiotics as Bacitracin, Penicillin, Chloramphenicol, Rifampicin, Vancomycin, Ciprofloxacin, Ofloxacin, Norfloxacin, and Tetracycline and resistance to Clindamycin, Erythromycin, Oxicillin, Nalidixic acid, and Lincomycin **(Table 3)**.

The characteristics of isolate HwSw11 were compared with reference strains (*Thermoactinomyces vulgaris*, *Thermoflavimicrobium dichotomicum*, *Laceyella sacchari* and *Laceyella putida*) as appeared in Table (4). Regarding morphological, biochemical and physiological characteristics, isolate HwSw11 exhibit growth pattern similar to family of *Thermoactinomycetaceae*. This family of actinomycetes requires pH greater than 7.0 for germination and optimum pH is 8.0 to 10.0 and temperature over to 50 ºC [74]. Depend on the comparative studies, it may be reported that the targeted isolate is enclosed to genus of *Thermoactinomyces dichotomicus*. In this way, Yoon et al. [73] reported that the strain of *Thermoflavimicrobium dichotomicum* is able to grow up to 62 ºC with optimum temperature of 55 ºC.

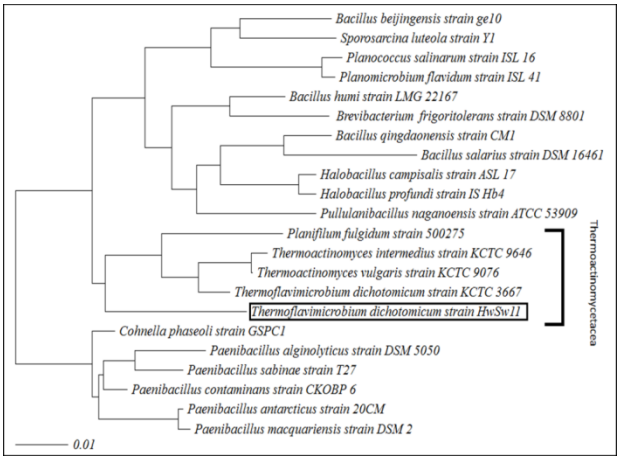


**Figure 5**. Phylogenetic tree of *Thermoflavimicrobium dichotomicum* HwSw11 with respect to reference strains based on the 16srRNA gene sequences.

The obtained identification was confirmed using molecular biology techniques. The partial 16S rDNA sequence (389 nucleotides) of actinomycetes HwSw11 was determined. The sequence was aligned and compared with sequences of the reference species available in BLAST genomic database, which confirmed that the HwSw11 isolate is closely related to *Thermoflavimicrobium dichotomicum* KCTC 3667T with identity of 94%. Multiple sequence alignment and phylogenetic tree were made as illustrated graphically in Figure (5). The phylogenetic tree was derived from the distance matrices using neighbor-joining [75]. So, upon classical and molecular identifications, this isolate was named *Thermoflavimicrobium dichotomicum* HwSw11 as a novel isolate and deposited in GenBank under this name and accession number of KR011193.

### 3.4. Antimicrobial activity.

Nowadays, the resistance mechanisms of almost pathogens are growing fast, because that the obtaining or research about new antimicrobial agents is considered the true way for controlling of these pathogens [5,9]. To achieve this goal, this study focused on isolation of new isolates from harsh environment. The obtained isolate, *Thermoflavimicrobium dichotomicum* HwSw11 strain, was screened for its ability to produce antimicrobial materials. The cell free supernatant of this strain was examined against some pathogens and found to be produced specifically antimicrobial agent. It has antibacterial activity against gram negative bacteria only like *P. aeruginosa* and *E. coli* with inhibition zones of 14 and 20 mm, respectively. Also, it has produced antifungal activity against *A. niger*, *A. flavus* and *Penicillium* sp. with inhibition zones of 17, 14 and 14 mm, respectively (**Table 4**).

**Table 4.** A comparative study of the identification properties of the isolate HwSw11 in relation to the reference strains

| Isolates / Characters | HwSw11 | *L. putida* | *L. sacchari* | *T. vulgaris* | *T. dichotomicum* |
|---|---|---|---|---|---|
| **Morphological Characteristics** | | | | | |
| Aerial mycelium* | Hygro-scopic | White | White | White | yellow |
| Substrate mycelium* | Orange | Yellowish-brown | Olive-buff | White or cream | Orange |
| Spore surface | Ridged | Smooth | Ridged | Rough | Ridged |
| Motility | Motile | Motile | Motile | Motile | Motile |
| Diffusible pigments | Yellow | Yellow | Yellow | No | Yellow |
| **Physiological Characteristics** | | | | | |
| Melanin pigment | - | + | + | - | - |
| Nitrate reduction | - | nd | nd | nd | - |
| Gelatin liquefaction | + | + | + | + | + |
| Optimum temp. ºC | 55 | 48 | 55-60 | 55 | 55 |
| Optimum pH | 9 | 7.2-7.4 | 7.2-7.4 | 7.2-7.4 | 7.2-7.4 |
| NaCl % tolerance | 1 | <1 | 1 | 1-5 | 0.5 |
| **Enzyme activities** | | | | | |
| Amylase | + | + | + | - | + |
| Cellulase | + | nd | nd | nd | - |
| Casienase | + | + | + | + | + |
| Lipase | + | + | + | + | + |
| **Utilizations of carbon sources** | | | | | |
| D-glucose | + | + | + | + | + |
| L-arabinose | + | nd | nd | nd | + |
| Sucrose | + | nd | nd | nd | + |
| D-xylose | + | nd | - | - | + |
| D-maltose | + | + | + | + | + |
| Cellulose | + | nd | nd | nd | - |
| Starch | + | nd | nd | nd | + |
| I-inositol | + | nd | nd | nd | + |
| D-mannitol | + | - | + | + | + |
| D-fructose | + | - | + | + | nd |
| Rhamnose | + | - | - | - | + |
| Raffinose | + | - | + | nd | + |
| Lactose | + | - | + | - | + |
| **Chemotaxonomic analysis** | | | | | |
| Diaminopimelic acid (DAP) | Meso-DAP | Meso-DAP | Meso-DAP | Meso-DAP | Meso-DAP |
| Sugar pattern | ND | ND | ND | ND | ND |

*Aerial and substrate mycelium colors were observed on CYC agar medium; ND: not detected; nd: not determined

On the other hand, it did not have antimicrobial activity against the tested gram positive bacteria and yeast pathogens. In the present study, we targeted the isolation of actinomycetes because it produces about 70-80 % of the available natural antibiotics in the world. The chance is the discovery of strains of

rare actinomycetes producing novel antimicrobial compounds [76].

**Table 5.** Antimicrobial activity of *Thermoflavimicrobium dichotomicum* HwSw11 supernatant

| Test organisms | | Inhibition zone diameter (mm) |
|---|---|---|
| Bacteria | ***B. cereous*** | 0 |
| | ***S. aureus*** | 0 |
| | ***P. aeruginosa*** | 14 |
| | ***E. coli*** | 20 |
| Fungi and Yeast | ***C. albicans*** | 0 |
| | ***A. niger*** | 17 |
| | ***A.flavus*** | 14 |
| | ***Fusarium sp.*** | 0 |
| | ***Penicillium sp.*** | 14 |

### 3.5. Optimization of beneficial enzymes production.

The old strain of *Thermoflavimicrobium dichotomicum* hasn't produce cellulases enzymes and cannot degrade cellulose compounds as the largest agriculture waste [41, 73]. Fortunately, the novel *Thermoflavimicrobium dichotomicum* HwSw11 showed great production of many useful enzymes like amylase, cellulase, lipase, pectinase and proteinase on solid agar medium. For that, the optimization of enzymes production was the target in this section. The main objective in this method is the optimum condition will be allowed only when achieving the microbial growth with production of targeted enzymes together.

In the case of optimum growth media, *Thf. dichotomicum* strain HwSw11 was inoculated on 9 different media and the growth plus enzymes assay were observed after 48 h. The results were recorded by values; weak, moderate, good, very good as represented in **Table (6)**. The results showed that the medium producing the highest growth and enzyme activity at the same time was ISP2 medium.

**Table 6.** Effect of culture media on the growth and enzymes production of *Thf. dichot-omicum* strain HwSw11

| Media | Growth | Enzymes production |
|---|---|---|
| Starch-nitrate agar medium | Moderate | Moderate |
| Yeast extract-malt extract agar medium (ISP-2) | Very good | Very good |
| Oatmeal agar (ISP-3) | Weak | Weak |
| Inorganic salts- starch agar medium (ISP-4) | Good | Moderate |
| Peptone yeast extract-iron agar medium (ISP-6) | Good | Moderate |
| Nutrient agar | Very good | Good |
| Marine agar | Weak | Weak |
| Plate count agar medium | Very good | Moderate |
| Basal mineral salts agar | Weak | Weak |

Also, the incubation period is a crucial factor for enzyme production, strain biomasses were collected every 12 h for a period of 96 h and enzymes assessed. The results showed that the best incubation period to produce the highest biomasses wasobserved after 36 h of incubation (**Fig. 6**).

Thermoalkaliphilic actinomyces species are generally used as a source of thermo-stable enzymes. It isn't only heat resistant, but also stable under highly alkaline conditions [77]. *Thf. dichotomicum* strain HwSw11 was grown at different temperatures ranged from 15 to 70 ºC. *Thf. dichotomicum* strain HwSw11 was able to grow at the temperature ranged between 35 and 60 ºC, but the optimum temperature for growth and producing all enzymes at high levels were 55 ºC (**Fig. 7**).

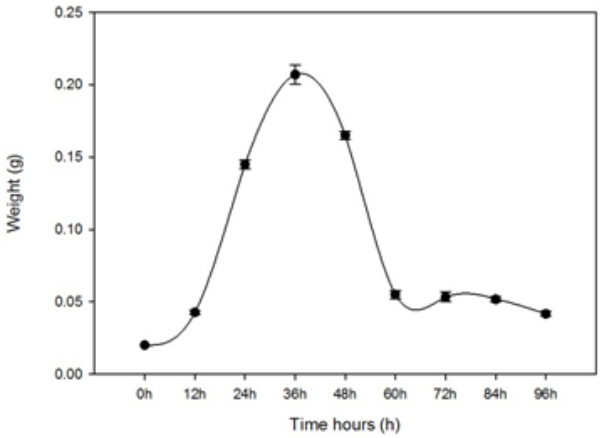


**Figure 6.** Optimization of incubation period (ISP2 broth, 55ºC) for *Thf. dichotomicum* strain HwSw11.

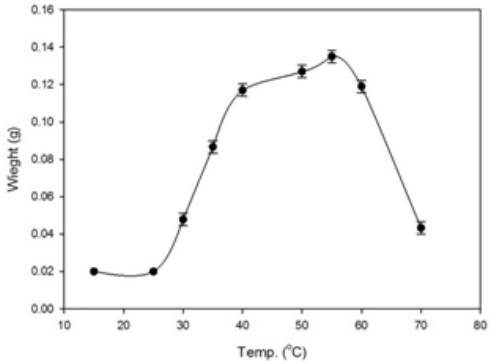


**Figure 7**. Optimization of incubation temperature (ISP2 broth, 36 h) for *Thf. dichotomicum* strain HwSw11.

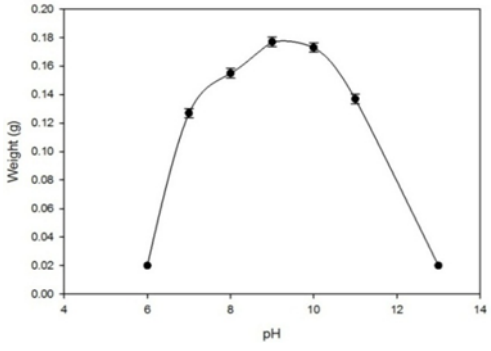


**Figure 8**. Optimization of initial pH (ISP2, 55 ºC, 36 h) for *Thf. dichotomicum* strain HwSw11.

Another important factor for actinomyces growth or enzymes and other bioactive compounds production is the initial pH value of cultivation media [78]. In this way, *Thf. dichotomicum* strain HwSw11 was cultivated into media adjusted to different pH values ranged from 6 to 13. The strain of HwSw11 exhibited the highest biomass and enzymes production was recorded when it cultivated at pH 9 (**Fig. 8**).

The produced enzymes by *Thf. dichotomicum* HwSw11 were had a lot of useful applications in environmental, agricultural and industrial area. It has advantage instead other enzymes as it is thermoalkaliphile enzymes and resistant to a wide range of environment conditions. Protease, pectinase and cellulases can be

applied in agricultural field for plant pathogens controlling as safe antifungal and antinematodal agents [79, 80]. Also, these enzymes used for bioenergy production from agricultural wastes by degrading agricultural wastes as ecofriendly agents [81, 82]. On the other way, lipase and amylase can be used in industries like food and detergent industry [83].

## 4. CONCLUSIONS

Based on the obtained results, a novel *Thermoflavimicrobium dichotomicum* HwSw11 as thermoalkaliphile actinomycete was isolated from Siwa oasis, Egypt. The isolated actinomycete exhibited extraordinary ability to produce several valuable enzymes including; amylase, cellulase, lipase, pectinase and proteinase. In addition, it produced noticeable anti-microbial agents against several serious pathogens. For that, the obtained strain may be a multiple factories for production useful new bioactive materials to be applied in several industrial, biotechnological, environmental and agricultural fields.

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## 6. ACKNOWLEDGEMENTS

The authors wish to thank the contribution between Al-Azher University, Cairo, Egypt, Agricultural Microbiology Department, National Research Centre, Giza, Egypt and School of Material Sciences and Engineering, Hebei University of Technology, Tianjin, China.