Iranian Journal of Organic Chemistry Vol. 9, No. 3(2017) 2111-2116

Isolation and identification of *Bacillus subtilis* producing biosurfactant and study the optimum condition of biosurfactant production

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Received: May 2017; Revised: May 2017; Accepted: July 2017

Abstract: 102 bacterial isolates were obtained from 40 oil contaminated soil samples from various automobile workshops, cars fuel stations, oil refineries of five different areasinIraq. All these isolates were subjected , to molecular identification based on the 16S rDNA by two sets of primers (B16SF and B16SR),(27Fand1492R),from these isolates 51 of the identified isolates belonged to *Bacillus subtilis*.Maximumbiosurfactant production from these isolates was found in 8 isolates symbol (S1,S2,S3,S4,S5,S6,S7,S8) , it was achieved by using olive oil,ammonium sulfate,at pH 7.0 ,30ºC and incubation period for 72 hrs.

Keywords: *Bacillus subtilis*, PCR, Biosurfactant, Optimum condition.

Introduction

Biosurfactants are active compounds are of two types ,chemically synthesized (surfactants) and biologically synthesized (biosurfactants), the chemical surfactants are produced from petrochemicals and are considered to be environmentally unsafe because they can lead to an ecological imbalance due to less biodegradability and toxicity [1].The main classes of biosurfactantshave been isolated from hundreds of microorganisms including lipopeptides, glycolipids, lipoproteins, phosphorlipids, fatty acids, polymeric surfactants and particulate surfactants [2,3]. Antimicrobial peptides (AMPs) are widespread synthesized by bacteria of the genus *Bacillus*. Among*Bacillus*, the production of AMPs has been recognized For *B.subtilis*, *B cereus*, *B.thuringiensis*, and *B. licheniformis*, but they were also reported in many other *Bacillus* species [4-6].

Results and Discussion

Isolation of biosurfactant-producing Bacillus species: For isolate *Bacillus* producing biosurfactant, 40 samples of heavily oil contaminated soil were collected from automobile work shop, cars fuel stations, oil refineries and surface beneath electrical machine ,of different area,from which 102 bacteria were isolated as shown in (Table **1**) .Oil contaminated soil were selection for isolation of biosurfactant producing *Bacillus* so they enhanced bacteria to utilize hydrocarbons in soil as nutrition source [11].The obtained isolates 102 were examined ,80 isolates suspected to be *Bacillus*spp.,and subjected to 16S rDNA analysis. Results indicated 51 isolates of the identified isolates belonged to*B.subtilis*, all 51 isolates were screened to determine the ability of this species to produce bio surfactant.

Molecular Identification of Biosurfactant producing Bacillus species:

The extracted DNA samples of the selected isolates were used to amplify16S rDNA for local isolates of *Bacillus* spp., the purity of DNA samples were analyzed by gel electrophoresis, These identification results are shown in (Figures **1**, **2**) which confirmed that the *Bacillus* isolates returned to *B.subtilis* .PCR products for the selected isolates were observed on UV

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transilluminator. The result showed an amplicon band for each one with the size of 1500 bp. The other set of primer (B16SF and B16SR), which was the hypervariant region (HV region) in the16S rDNA gene, the region was highly specific for each type of bacterial strain of *Bacillus* spp. and were used to amplify the 5' end region approximately 275 bp PCR products for the selected isolates were observed on agarose gel electrophoresis showed an amplicon band for each one with the size of 275bp respectively [12].

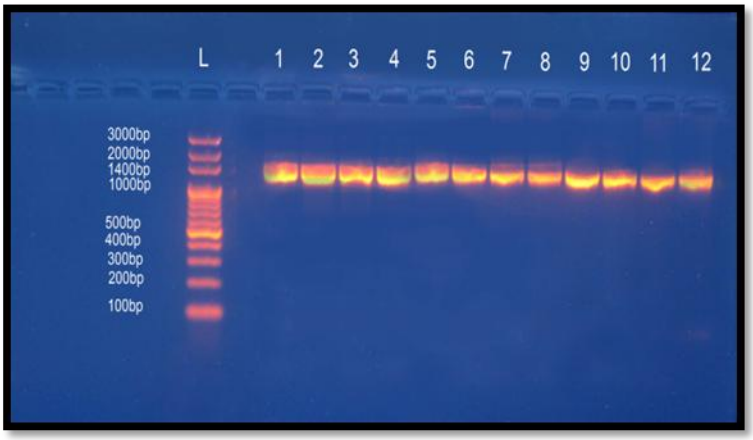

Figure 1: PCR molecular size products of 16S rDNA by the set of primers (27F and1492R) of the *Bacillus* isolates.

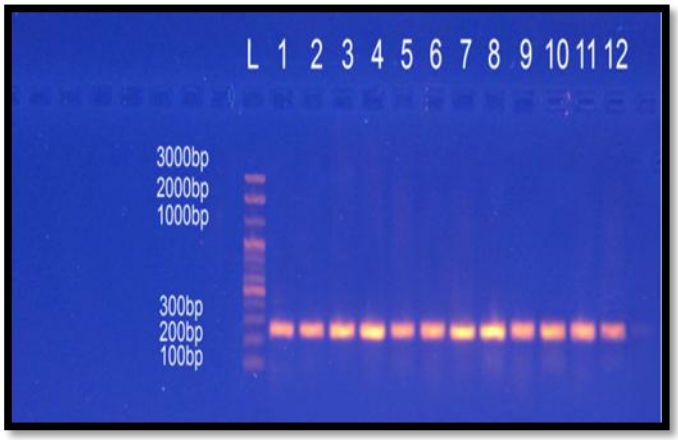

Figure 2: PCR molecular size products of 16S rDNA by the set of primers (B16SF and B16SR) of the *Bacillus* isolates.

Partial purification of biosurfactant produced by the selected isolates of B.subtilis:

Optimization of biosurfactant production by isolate B. subtilisS4:

Effect of carbon source:

The important factor in biosurfactant production was carbon source (Figure **3**). The results mentioned above showed that olive oil was the best substrate for production of biosurfactant it gave 0.27g/l, followed by glucose, it was reached to 0.24 g/l. Olive oil was the best carbon source for surfactant synthesis the reason for this tendancy of *B.subtilis* is lipase fatty acids contained in olive oil fractions and which have the ability to significantly enhance the degradation of any of the oils used [13].

Effect of nitrogen source:

In organic nitrogen source ammonium sulfate were more efficient in enhancing the isolate for the production of biosurfactant which caused the production of highest yield of reached to 0.23g/l followed by potassium nitrate in the amount production of it gave 0.13g/l, ammonium nitrate and sodium nitrate were less efficient in producing biosurfactant positive which facilitate the assimilation of reaching dry weight for both to 0.1 g/l**.**

Table 1: Weight of partially purified biosurfactant from the selected *Bacillus subtilis.*

Isolated Symbol	Incubation periods (hrs.)	Partially purified biosurfactant (g/l)
S1	48	0.12
S2	72	0.09
S3	72	0.1
S4	72	0.18
S5	72	0.14
S6	48	0.1
S7	72	0.09
S8	72	0.07

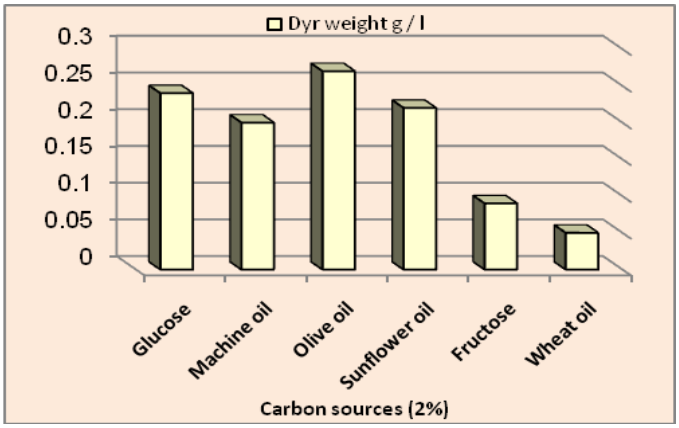

Figure 3: Effect of carbon source (2%) on biosurfactant production from *B. subtilis*

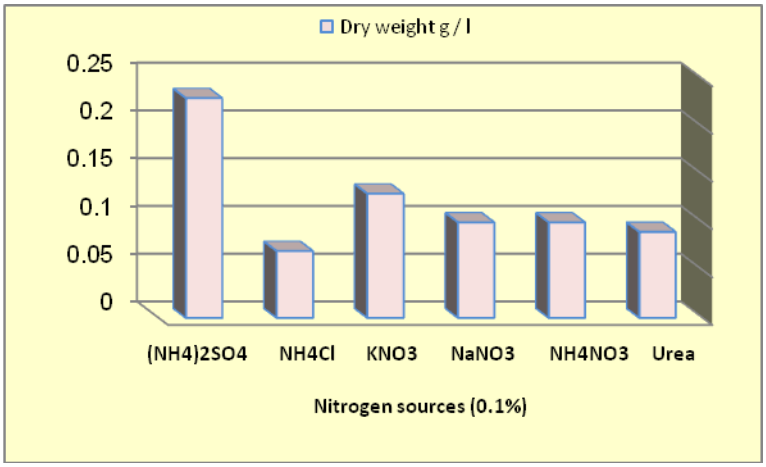

Figure 4: Effect of nitrogen source (0.1%) on biosurfactant production from *B. subtilis*S4.

These results were in accordance with other study found that ammonium sulphate was the best nitrogen source which enhanced *B.subtilis*HOB2 for biosurfactant production in the medium.

Effect of PH:

Different ranges of pH medium were used for testing to optimize the production of biosurfactant, pH of medium plays an important role in biosurfactant production. These results as seen in (Figure **5**) observed that the optimum pH for was 7 reaching dry weight to 0.29 g/l followed by pH6.0 it was reaching to 0.24 g/l and the yield drop when pH decreased or increased from that value. Similar study it was demonstrated that optimum biosurfatant production from *B.cereus* HI-2 obtained at pH 7.0 (Kadhim, 2006). It was found that the type, quantity and quality of biosurfactant produced are influenced by culture conditions such as pH (Karanth et al.,1999).

Effect of Temperature:

The optimal incubation temperature was 30°C for bio surfactant production it reached to 0.28 g/l as seen in (Figure **6**) followed by 35 °C it was reaching to 0.25 g/l and dropped at 25ºC and 40ºC .

Effect of incubation period:

Results in (Figure **7**) illustrated that dry weight of surfactin increasing with the increased in incubation period up to 72 hrs, it was reaching to 0.3 g/l, followed by 48 hrs it was recorded 0.26 g/l above and below of incubation period 72 hr biosurfactant concentration was decreased . In other study demonstrated that in *B.subtilis*the surfactant biosynthesis on cassava waste started at exponential growth phase and continued during the stationary phase, about 50% of biosurfactant was produced when cell growth reached stationary phase.

Experimental

Objectives of study:

1. Isolation of *Bacillus* spp. from different localities contaminated with hydrocarbons.
2. Identification of *Bacillus subtilis* and selection of efficient isolates that screening for their ability to produce biosurfactant .
3. Determination of the optimum conditions for biosurfactant production.

Methods:

Soil samples collection:

Soil samples were collected from different locations of Najaf, Kufa and Diwaniya governorates .Samples were taken from the oil spilled surface of the automobile workshop [7]. **2.2Isolation of Bacteria**

Isolation of *Bacillus* spp. was performed by by adding one gm from each soil sample to 9 ml of sterile D.W. , mixed well and then heated in water bath at 80ºC for 20 min ,with gentle agitation, after cooling spreaded on a nutrient agar andincubated for 24 hrs.at 30°C [8].

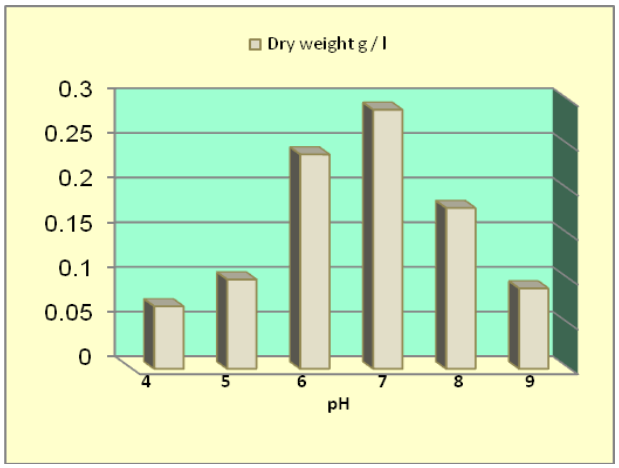

Figure 5: Effect of pH on bio surfactant production from *B. subtilis* S4.

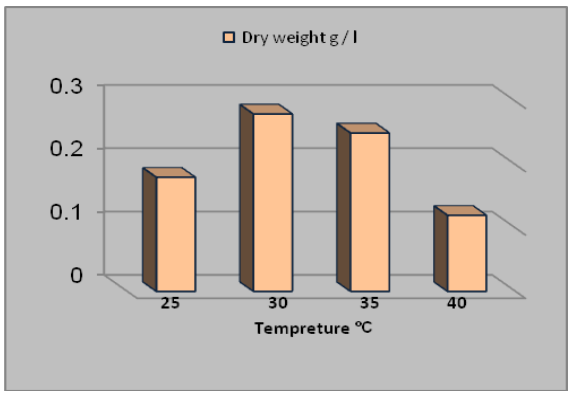

Figure 6: Effect of temperature on biosurfactant production from *B.subtilis* S4.

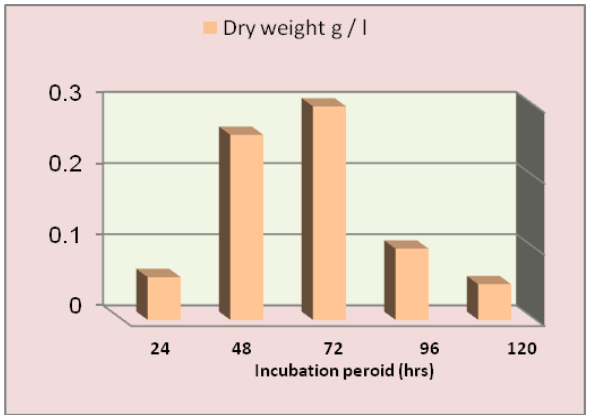

Figure 7: Effect of incubation period on biosurfactant production from *B. subtilis*S4.

Molecular Identification:

Extraction of Genomic DNA;

One bacterial colony age 24 hrs was scraped using sterile loop from surface of agar plates and suspended in 40 µl Tris-EDTA buffer . The suspension was heated for 15 min at 100 ºC followed by 5 min on ice directly , the suspension containing DNA was stored at ˉ20ºC until used as template for PCR .

Primers Selection:

Table **1** Sequences of primers used to amplify the 16S rDNA sequence of bacterial isolates.

Detection of DNA Content by Agarose Gel Electrophores:

Gel electrophores was used for detection of DNA by UV transilluminator [9].TBE (1X) buffer was poured to the electrophoresis tank,tray with agarose gel was immersed in electrophoresis tank .Each well was loaded with 5µl of DNA sample and standard molecular weight of DNA ladder marker) was loaded in afirst well. Electrophoresis run at 80 volts for 1hr , gel was visualized under UV transilluminator and photographed by using digital camera [10].

Biosurfactant production:

One ml of the inoculums was used to inoculate 100 ml of Jacques medium , in triplicate, and incubated aerobically in shaker incubater at 180 rpm at 30ºC for three periods (24,48and72hrs).Each culture was centrifuge at 4 ºC, 10.000 rpm. for 20 min .

Optimization of biosurfactant production:

Effect of carbon sources:

Six carbon sources (Glucose, Fructose, Olive oil, Machine oil,Wheat oil and Sunflower oil),were used to determine the optimum carbon source for production by the selected isolate of *Bacillus* spp. Each of these carbon sources was added to the medium in a concentration of 2% (w/v).

Effect of nitrogen source:

Six nitrogen sources (NH4NO3 ,(NH4) 2SO4 ,NH4Cl, KNO3, NaNO3, and Urea),were used to determine the optimum nitrogen source for biosurfactant production.These nitrogen sources were added to the production medium in a concentration of 0.1% (w/v) or (v/v) .

Effect of medium pH:

The production medium was adjusted to different pH values [4-9] to determine the optimum pH for biosurfactant production by using HCL as a acid and NaOH as a base.

Effect of temperature:

The production medium was incubated at different temperatures (25, 30, 35 and 40ºC), in order to determine the optimum temperature for production of bio surfactant.

Effect of incubation period:

For study the effect of incubation period of *Bacillus* spp in production, medium was incubated for 24, 48, 72, 96 and120 hrs.

Primer type		Primer Sequence (5'-3')	Sizebp	Tm(ºC)
27	F	5′-AGAGTTTGATCCTGGCTCAG-3′	20	61
1492	R	5′-ACGGTTACCTTGTTACGACTT-3′	21	59
B16S	F	5′-TGTAAAACGACGGCCAGTGCC TA ATACATGCAAGTCGAGCG-3′	41	60
B16S	R	5′-CAGGAAACAGCTATGACCACTG CTGCCTCCCGTAGGAGT-3′	39	60

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