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Research Article
Isolation, Purification and Characterization of Antimicrobial Agent Antagonistic to *Escherichia coli* ATCC 10536 Produced by *Bacillus pumilus SAFR-032* Isolated from the Soil of Unaizah, Al Qassim Province of Saudi Arabia

[1]Abdurrahman S. Alanazi, [2]Kamal Ahmad Qureshi, [2]Gamal Osman Elhassan and [3]Elsayed I. El-Agamy

[1]Department of Pharmacy Practice, Unaizah College of Pharmacy, Qassim University, Kingdom of Saudi Arabia
[2]Department of Pharmaceutics and Pharmaceutical Chemistry, Unaizah College of Pharmacy, Qassim University, Kingdom of Saudi Arabia
[3]Department of Applied Medical Sciences, Unaizah Community College, Qassim University, Kingdom of Saudi Arabia

Abstract

Background: *Escherichia coli* is one of the most common pathogenic bacteria, which cause urinary tract infections in infants as well as in adult human beings. Due to the emergence of antibiotic resistance in *E. coli*, there is a great demand of new antimicrobial agent for the treatment of infections caused by such *E. coli*. This study aims to isolate, identify and characterize the native soil-bacterial strains predominate in the soil of Unaizah city, which produce antimicrobial agent antagonistic to *E. coli* ATCC 10536, followed by isolation, purification and characterization of antimicrobial agent. **Materials and Methods:** Pour plate, spread plate and 16S rRNA sequence analysis methods were followed for the isolation and identification of soil bacteria. Ammonium sulphate and dialysis (MWCO-8 KD) methods were followed for the isolation and partial purification of antimicrobial agent from the cell free broths. The characterization of antimicrobial agent was carried out by determining the minimum inhibitory concentration and effects of temperature and pH on the antimicrobial stability. **Results:** Out of the twenty five soil samples, only one soil-bacterial strain was found to produce antimicrobial agent antagonistic to *E. coli* ATCC 10536. The isolated soil bacterium was identified as *Bacillus pumilus* SAFR-032. The soil isolate was characterized and results suggest that 30°C temperature and pH 7.0 were the optimum growth parameters and soybean casein digest broth was the best fermentation medium, whereas the highest production of antimicrobial agent was at 35°C temperature, pH 7.0, shaking at 150-220 rpm and at 60th h of incubation. The maximum yield of antimicrobial agent was obtained at 60% of $(NH_4)_2SO_4$. The results of characterization of antimicrobial agent suggest that the maximum and minimum antimicrobial activities were at pH 3.0 and 8.0, respectively, whereas antimicrobial activity was unaffected by temperature. The antimicrobial agent was highly stable at varying range of temperature 50-120°C. Minimum inhibitory concentration of antimicrobial agent was found to be 64 μg mL^{-1}. **Conclusion:** In conclusion, this study might be a great endeavor for the healthcare industry in order to treatment of different infections caused by *E. coli* and that warrants further investigations to fully standardized and establish the antimicrobial profile of effect(s) of this isolate.

Key words: Antimicrobial agents, *Bacillus pumilus* SAFR-032, ammonium sulphate method, *E. coli* ATCC 10536

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Corresponding Author: Kamal Ahmad Qureshi, Department of Pharmaceutics and Pharmaceutical Chemistry, Unaizah College of Pharmacy, Qassim University, Kingdom of Saudi Arabia Tel: +966599110591

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Escherichia coli is a non-pathogenic normal flora of human intestine. They comprise around 10^6-10^9 Colony Forming Units (CFU) per gram of fecal sample[1]. The organism is also found in natural environments like water and soil, usually as a result of stool contamination. Several strains of *E. coli* have been described, which cause infections in gastrointestinal system, while other strains cause infections outside the gastrointestinal system[2]. It is one of the most common causative agents of Urinary Tract Infections (UTIs) in humans[3] and is a leading causative agent of intestinal as well as systemic infections (bacteremia, cholecystitis, cholangitis, cellulitis, osteomyelitis, nosocomial pneumonia, peritonitis, cellulitis, infectious arthritis, osteomyelitis and neonatal meningitis)[4].

This pathogenic bacterium is responsible for a wide range of hospital and community-onset infections and also affects the patients with normal immune systems as well as those with poor immune systems[5]. They often comprise the most common Gram Negative Bacteria (GNB) found in clinical laboratory settings including the wide majority of urinary, blood, skin and peritoneum isolates. It may also cause infections in other parts of body including the cerebrospinal fluid, respiratory tract and various types of abscesses. Multi drug resistant isolates, especially resistant to fluoroquinolones and those producing extended-spectrum β-lactamases (ESBL) have increased significantly during the last decade and therefore, many nosocomial and community acquired *E. coli* are now resistant to several important antibiotic classes[5].

Antimicrobial agents are the chemical compounds produced by living cells (microorganisms, plant, animal etc.), which inhibit the growth of other organisms at very low concentrations. They may be either static (inhibit the growth of other microorganisms) or cidal (completely kill other microorganisms). The soil microorganisms (Actinobacteria, Eubacteria, Fungi) are the most important source of antimicrobial agents. The antimicrobial agents produced by soil eubacteria might be either primacy metabolites (bacteriocins) or secondary metabolites (antibiotics)[5].

Bacteriocins are a type of ribosomally synthesized antimicrobial peptides also known as proteinaceous toxins produced by eubacteria, which can kill or inhibit the growth of similar or closely related bacterial strains but will not harm the eubacteria themselves by specific immune proteins[6], whereas antibiotics (secondary metabolites) are the natural chemical molecules secreted by soil microbes and are inessential for the survival of microorganisms but provide a competitive advantage within their environment, whereas some have useful therapeutic properties[7-9]. Additionally, they facilitate symbiosis between insects, plants and higher animals[9].

Soil eubacteria have variety of strains, which produce antimicrobial agents. It has been reported that *Bacillus* species are one of the most common soil eubacteria, which produce various types of primary and secondary metabolites. *Bacillus* species are rod-shaped, endospore-forming aerobe or facultative anaerobe, Gram Positive Bacteria (GPB). They exhibit a wide range of physiologic abilities that allow them to live in any natural environment[9,10]. The spores of *Bacillus* species are resistant to heat, cold, radiation, disinfectants and desiccation. *Bacillus pumilus* is one of the predominant *Bacillus* species found in the natural environments (soil and water). It has been reported that *Bacillus pumilus* produces variety of antimicrobial agents, however, there is insignificant information available about the antimicrobial agent produced by *Bacillus pumilus* SAFR-032 antagostic to *E. coli*. Thus the present study aims to isolate, identify and characterize the native soil-bacterial strains, which produce antimicrobial agent(s) antagonistic to *E. coli* ATCC 10536 predominant in the soil of Unaizah city, followed by isolation, purification and characterization of antimicrobial agent(s).

MATERIALS AND METHODS

Preparation of test medium plates: Soybean casein digest agar was used as a test medium whereas, *E. coli* ATCC 10536 was used as a test organism. Each plate of test medium was prepared by adding 1 mL suspension of test organism (turbidity ~0.5 McFarland) for isolation of eubacteria from the soil samples.

Preparation and inoculation of soil samples: Twenty five soil samples were collected from the different regions of Unaizah, Al Qassim province of Saudi Arabia. Each soil sample was diluted in series by using sterile normal saline as a diluent thereafter 1 mL sample from each dilutions (10^{-4} and 10^{-5}) were inoculated into soybean casein digest agar plates, which were preinoculated with test organism[9-11].

Isolation, identification and characterization of soil isolate(s)

Isolation of soil eubacteria: The inoculated plates were incubated at 25-30°C for 48-120 h and thereafter, the isolated colonies were screened for antimicrobial activity and data was recorded. The desired isolated colonies were picked up and purified for further investigations.

Determination of antimicrobial activity of soil isolate(s) via spot inoculation: The antimicrobial activities of soil isolates were determined by spot inoculation on Mueller Hinton agar plate, which was pre-inoculated with 100 µL suspension of *E. coli* ATCC 10536. Thereafter, inoculated plate was incubated at 25-30°C for 48-120 h. The antimicrobial activities of soil isolate(s) were confirmed and results were recorded.

Identification of soil isolate: The soil isolate was selected on the basis of antimicrobial activity against the test organism and thereafter purification and labelling were done. The selected soil isolate was identified by phenotypic as well as genotypic (16S rRNA sequence analysis) methods. The replica culture plates of soil isolate were kept at 4°C temperature in glycerol for further analysis[9-15].

Characterization of soil isolate

Effects of ph on growth: The effects of pH were determined by incubating the bacterial suspensions at constant temperature (35°C) with different pH values starting from 2.0 up to 10 for 24 h. Thereafter, optical densities of all suspensions were measured at 600 nm whereas results were compared with an unincubated suspension.

Effects of temperature on growth: The effects of temperature were determined by incubating the bacterial suspensions at different temperatures starting from 20°C up to 50°C for 24 h whereas, the pH (7.0) was constant throughout the incubation. Thereafter, optical densities of all suspensions were measured at 600 nm, whereas results were compared with an unincubated suspension[16,17].

Optimization of fermentation conditions

Inoculum preparation: Five percent inoculum was prepared in soybean casein digest broth by using the master culture of *Bacillus pumilus* SAFR-032.

Selection of fermentation medium: In order to known the best fermentation medium, different types of nutrient media were used for fermentation purposes such as Nutrient broth, Luria broth and soybean casein digest broth. Each fermentation medium was seeded with 5% inoculum of soil isolate and then all seeded media were incubated at 30°C temperature with pH 7.0, shaking at 150-220 rpm for up to 120 h[14-17].

Cell Free Broth (CFB): The fermented broth samples were collected at every 12 h of incubation, starting from 0 h up to 120 h and then antimicrobial activity of each collected sample was measured by well diffusion method. All the collected broth samples were centrifuged at 5500 rpm at 4°C temperatre for 30 min and then supernatants were filtered through a 0.22 µm pore size filter and thereafter these cell free broths were analyzed for antimicrobial activity by well diffusion method[18].

Antimicrobial assay of cell free broths: In briefly, 8 mm diameter wells were cut off into Mueller Hinton agar plates, pre-inoculated with *E. coli* ATCC 10536 and then 80 µL quantities of each CFB were inoculated into each well. All inoculated plates were pre-incubated at room temperature for around 2-3 h, followed by incubation at 37°C temperature for 24-48 h. The diameter of each zone of inhibition was measured in millimeters and results were recorded.

Effects of temperature on the production of antimicrobial agent: Four flasks of soybean casein digest broth were seeded with 5% of inoculum at pH 7.0 and then broths were incubated at different temperatures such as 25, 30, 35 and 40°C with shaking at 150-220 rpm for up to 120 h. Thereafter, the broth samples were collected at every 12 h of incubation; starting from 0 h up to 120 h and then the antimicrobial activities of all cell free broths were determined by well diffusion method.

Growth curve of *Bacillus pumilus* SAFR-032: The growth curve was prepared by using the data obtained from the fermentation of soybean casein digest broth. The fermentation medium was seeded with 5% inoculum of soil isolate and then seeded medium was incubated at 35°C temperature with pH 7.0, shaking at 150-220 rpm for up to 120 h. The broth samples were collected at every 12 h of incubation, starting from 0 h up to 120 h and thereafter, the pH and optical density (at 600 nm) were measured whereas, the antimicrobial activity of each collected sample was determined by well diffusion method[19-22].

Isolation, purification and characterization of antimicrobial agent

Isolation and purification of antimicrobial agent: The antimicrobial agent was isolated from the cell free broths by ammonium sulphate method. The different concentrations of ammonium sulphate were used to isolate the antimicrobial agent from cell free broths[23-25].

Partial purification by dialysis: The ammonium sulphate salts were removed by dialysis method. The dialysis was carried out

by using cellulose membrane with MWCO: 8 KD and phosphate buffer with pH 7.0. The chemical estimation (Total protein gram per liter) of dialysate was carried out by Biuret method. The dialysate was kept at 4-6°C temperature for further investigations.

Characterization of antimicrobial agent

Effects of pH on antimicrobial agent: The effects of pH were determined by incubating the antimicrobial dialysate at 4-6°C temperature with different pH starting from 2 up to 10 for overnight and thereafter, antimicrobial activities of dialysates (each 80 µL) were determined by well diffusion method. The pH of antimicrobial dialysates were adjusted by using 1 N HCl and 1 N NaOH.

Effects of temperature on the antimicrobial agent: The effects of temperature were determined by incubating the antimicrobial dialysate at different temperatures with constant pH 3.0, starting from 50°C up to 120°C for 20 min and thereafter the antimicrobial activities of dialysates (each 80 µL) were determined by well diffusion method[26,27].

Determination of Minimum Inhibitory Concentration (MIC) by broth macro dilution method

Inoculum preparation: Around 5 pure culture colonies (24 h old culture) of test organism were picked up. The colonies were suspended in 5 mL of sterile saline (0.89% NaCl). The resulting suspension was vortexed for 15 sec and then cell density was adjusted equal to 0.5 McFarland standards at 600 nm wavelengths of spectrophotometer (absorbance ~0.08-0.10) by adding sufficient sterile saline. This cell density was equal to 1×10^8 CFU mL^{-1}. Optimally within 15 min of preparation stock suspension (0.5 McFarland), the stock suspension was diluted 1:150 in sterile Mueller Hinton broth, so the cell density reached around the 1×10^6 CFU mL^{-1}. Finally this cell density reached approximately 5×10^5 CFU mL^{-1} when it was diluted 1:2 in antimicrobial solution in the next step.

Antimicrobial agent weighting and dilution scheme: The powder form of antimicrobial agent was obtained by drying the dialysate at 80°C temperature for 30 min. Approximately 10 mg dry powder of antimicrobial agent was weighted and dissolved in 1 mL of methanol and vortexed well, now stock concentration of antimicrobial agent was equal to 10000 µg mL^{-1}. Thereafter, stock solution was further diluted in phosphate buffer (pH 3.0) to obtain the different concentrations, whereas augmentin 50 mg (Purity: >98%) was used as a standard antimicrobial agent. The dilution scheme was illustrated in Table 1 and 2.

Inoculation of broth medium: Before adjusting the inoculum, 1 mL of various concentrations of antimicrobial agent were placed in 12×75 mm tubes. The growth control tube was prepared by mixing of 1 mL distilled water with 1 mL of 1:150 diluted test organism suspension in Mueller Hinton broth without antimicrobial agent. Within 15 min after the inoculum has been standardized (up to 2 h if inoculum has kept at 4°C temperature), 1 mL of the adjusted inoculum (1×10^6 CFU mL^{-1}) was added to each tube in the dilution

Table 1: Antimicrobial agent dilution scheme (Macro broth dilution method)

Weight of powder (mg)	Volume of solution (mL)×Concentration of antimicrobial Agent (µg mL^{-1})/Potency of antimicrobial powder (µg mg^{-1})
Potency of powder (µg mg^{-1})	Volume of solution (mL)×Concentration of antimicrobial Agent (µg mL^{-1})/Weight of powder (mg)
Volume of solution	1 mL
Concentration of antimicrobial agent	10000 µg mL^{-1}
Weight of powder	10 mg
Potency of powder (µg mg^{-1})	1×10000/10 = 1000 µg mg^{-1}
Potency of antimicrobial agent	1000 µg mg^{-1}
(We have assumed the potency of partial purified antimicrobial agent as 100%)	
Volume of diluent (mL)	Weight of antimicrobial agent (mg)×Potency of antimicrobial Agent (µg mg^{-1})/Concentration of antimicrobial agent (µg mL^{-1})
Weight of antimicrobial agent	10 mg
Potency of antimicrobial agent	1000 µg mg^{-1}
Concentration of antimicrobial agent	1024 µg mL^{-1}
Volume of diluent (mL)	10×1000/1024 = 9.77 mL
Volume of diluent (mL)	9.77 mL
Thus 1 mL of stock solution (10000 µg mL^{-1}) was mixed with 8.77 mL of phosphate buffer (pH 3.0) for obtaining the final concentration 1024 µg mL^{-1} of antimicrobial agent	

Table 2: Antimicrobial agent dilution scheme (Macro broth dilution method)

Stock concentration of antimicrobial agent (μg mL^{-1})	Stock solution (mL)	Phosphate buffer (pH 3.0) (mL)	Intermediate concentration of antimicrobial agent (μg mL^{-1})	Final concentration of antimicrobial agent: 1:2 dilution (μg mL^{-1})
10000	1	8.77	1024	512
1024	1	1	512	256
1024	1	3	256	128
1024	1	7	128	64
128	1	1	64	32
128	1	3	32	16
128	1	7	16	8
16	1	1	8	4
16	1	3	4	2
16	1	7	2	1
2	1	1	1	0.5
2	1	3	0.5	0.25
2	1	7	0.25	0.125

series and mixed well. This resulted 1:2 dilution of each antimicrobial concentration and 50% dilution of the inoculum. Thus the final concentration of cell density was 5×10^5 CFU mL^{-1}. All the test tubes were kept in incubator at 37°C temperature for 24 h. The MIC results of antimicrobial agent were compared with the results of standard antimicrobial agent. The amount of growth in the tubes containing the antimicrobial agent was compared visually with the amount of growth in the growth-control tube used in each set of test[28,29].

RESULTS

Isolation, identification and characterization of soil isolate(s)

Isolation of soil eubacteria: Out of 25 soil samples, only one soil isolate was found to have antimicrobial activity antagonistic to *E. coli* ATCC 10536. The isolated soil strain was purified for further analysis.

Determination of antimicrobial activity of soil isolate(s) via spot inoculation: The results of antimicrobial activity of soil isolate suggest that the isolated soil strain was highly antagonistic to test organism *E. coli* ATCC 10536 thereafter, a temporary code: KA 18-B was assigned to this soil isolate.

Identification of soil isolate: KA 18-B: The soil isolate: KA 18-B was identified by both-phenotypic as well as genotypic methods.

Phenotypic characteristics: The morphological characteristics suggest that the soil isolate was Gram positive, aerobic, motile, non-capsulated, spore forming bacillus, whereas colony characteristics were as 1-1.5 mm in diameter, circular, elevated, smooth, convex, entire, γ-haemolytic and smell-earthy. The biochemical characteristics were illustrated in Table 3. The results were analysed and compared by using Bergey's manual of systematic bacteriology-volume 3.

Genotype characteristics: The genotypic identification was carried out by 16S rRNA sequence analysis. Around 700 bp sequences were analyzed. The 16S rRNA gene sequence results were analyzed with the GenBank using BLAST (GenBank). The accession code for NCBI database was NC_009848. The result of 16S rRNA gene sequence analysis is enlisted here with:

Sequence text (in FASTA format, 5' -> 3'):
CGGCGTGCCTAATACATGCAAGTCGAGCGGACAGAAGGGAGCTTGC
TCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG
CCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGAT
AGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTG
TCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGGGGTAA
TGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGG
GAAGAACAAGTGCGAGAGTAACTGCTCGCACCTTGACGGTACCTAA
CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAG
GGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGA
ATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA
GTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAA
AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTG
CAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGAC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGAC
ATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGACAGAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCA
TTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACCGCAAGG
TTTAGCCAATCCCATAAATCTGTTCTCAGTTCGGATCGCAGTCTGCA
ACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG

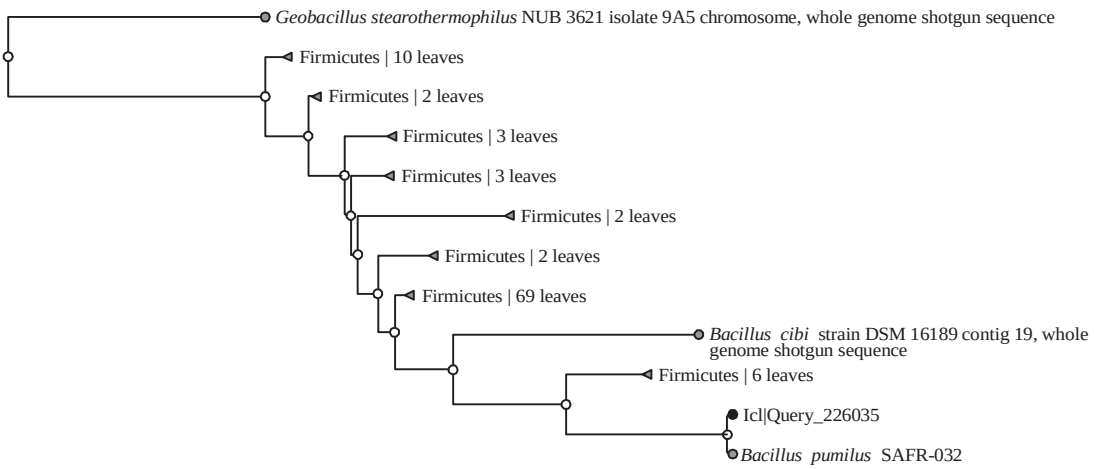

Fig. 1: Phylogenetic tree of *Bacillus pumilus* SAFR-032

Table 3: Biochemical characteristics of *Bacillus pumilus* SAFR-032

CV*	-	INU*	-
MS*	+	NACL*	-
NIT*	+	PRV*	-
NOV*	-	RAF*	-
MAN*	-	RBS*	-
LAC*	+	SOR*	-
TRE*	+	ARG*	-
MNS*	+	PGT*	+
ARA*	-	URE*	-
BAC*	+		

+: Test positive, -: Test negative, CV: Crystal violet, MS: Micrococcus screen, NIT: Nitrate, NOV: Novobiocin, PGR: PNP-β-D-Glucuronide, IDX: Indoxyl phosphatase, VP: Voges-proskauer, OPT: Optochin, PHO: Phosphatase, BE: 40% Bile esculin, PYR: L-pyrrolidonyl-β-naphthylamide, ARG: Arginine, PGT: PNP-β-D-Galactopyranoside, URE : Urea, MAN: Mannitol, LAC: Lactose, TRE: Trehalose, MNS: Mannose, ARA: Arabinose, BAC: Bacitracin, INU: Inulin, NACL: Sodium chloride 6.5%, PRV: Pyruvate, RAF: Raffinose, RBS: Ribose, SOR: Sorbitol

Thus results obtained from phenotypic as well as genotypic analysis suggest that the isolated soil bacterium KA 18-B has 99% similarity with *Bacillus pumilus* SAFR-032. The phylogenetic tree of *Bacillus pumilus* SAFR-032 was illustrated in Fig. 1.

Characterization of *Bacillus pumilus* SAFR-032

Effects of ph on growth: The results suggest that the optimum growth of soil isolate was obtained at pH 7.0.

Effects of temperature on growth: The results suggest that the optimum growth of soil isolate was obtained at temperature 30°C. Thus the results suggest that the optimum growth conditions of soil isolate were as pH 7.0 and temperature 30°C.

Optimization of fermentation conditions

Selection of best fermentation medium: The highest antimicrobial activity (zone of inhibition) produced by fermented Soybean casein digest broth, Luria broth and Nutrient broth were as 14, 12 and 10 mm, respectively at the end of 60th h of incubation, therefore the results suggest that the soybean casein digest broth was the best whereas Nutrient broth was the worst fermentation medium. Thus Soybean casein digest broth was selected for further fermentation process.

Effects of temperature on the production of antimicrobial agent: The results of antimicrobial activities (zone of inhibition) produced by fermented Soybean casein digest broths at different temperatures 25, 30, 35 and 40°C were as 10, 12, 14 and 12 mm, respectively at the end of 60th h of incubation. The highest antimicrobial activity (zone of inhibition) was 14 mm at 35°C temperature. Thus the results suggest that the highest yield of antimicrobial agent can be obtained at 35°C temperature because 35°C temperature was the optimum temperature.

Growth curve of *Bacillus pumilus* SAFR-032: The results of growth curve of soil isolate suggest that the initial pH was declined from 7.0-6.0 during the log phase and then slightly increased around 6.5 during the stationary phase and thereafter, it increased to 8.0 during the beginning of death phase and returned to 7.0 during the death phase. The bacterial growth was increased from 0.05-0.34 OD during the log phase and was constant around 0.34 OD throughout the stationary phase and then it increased from 0.34-0.40 OD during the beginning of death phase and followed by continuous declined during the death phase.

The antimicrobial activity (zone of inhibition) was 12 mm during the log phase and was constant 12 mm during

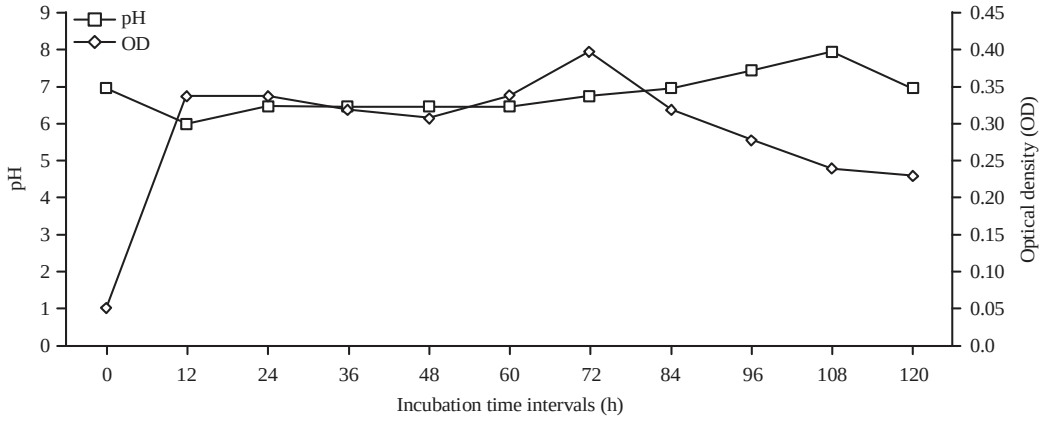

Fig. 2: Growth curve of *Bacillus pumilus* SAFR-032 (Incubation time vs. OD and pH)

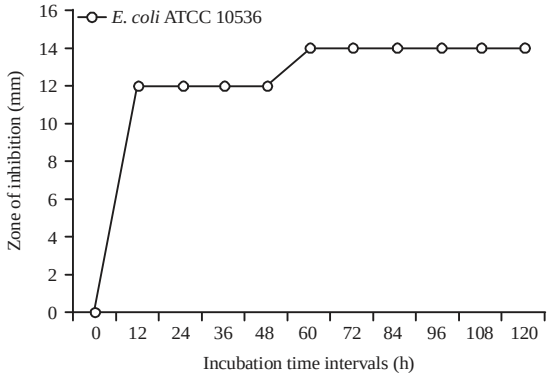

Fig. 3: Growth curve of *Bacillus pumilus* SAFR-032 (Incubation time vs. antimicrobial activity)

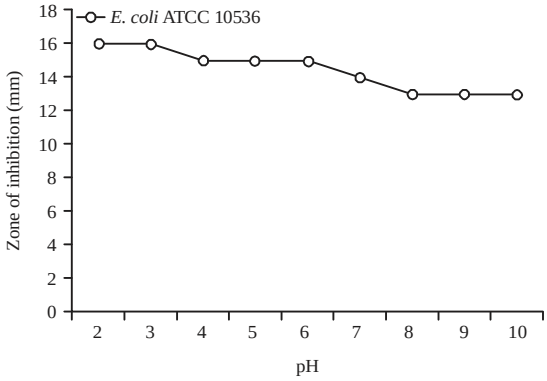

Fig. 4: Effects of pH on antimicrobial agent

the stationary phase and then it was increased to 14 mm during the end of stationary phase and was constant 14 mm during the death phase. Thus the maximum production of antimicrobial agent was during the end of stationary phase (60th h of incubation). The results[30] were illustrated in the Fig. 2 and 3.

Isolation, purification and characterization of antimicrobial agent

Isolation and purification of antimicrobial agent: The results suggest that the antimicrobial agent was successfully isolated by ammonium sulphate method and then partially purified by dialysis using 8 KD MWCO dialysis tubing and phosphate buffer (pH 7.0). Furthermore, the results suggest that the maximum yield of antimicrobial agent was at 60% concentration of ammonium sulphate and suggested molecular weight of isolated antimicrobial agent was not less than 8000 Daltons whereas the total protein of dialysate (concentration of isolated antimicrobial agent) was 5 g L^{-1} at 60% concentration of ammonium sulphate, which was highest yield.

Characterization of antimicrobial agent

Effects of pH: The results suggest that the pH affects the antimicrobial activity of dialysate (antimicrobial agent) as the maximum antimicrobial activity (zone of inhibition) was 16 mm at pH 3.0 and minimum antimicrobial activity (zone of inhibition) was 13 mm at pH 8.0. So the antimicrobial activity was increased at decreased pH values and antimicrobial activity decreased with increased pH values. Thus the pH was very important factor for the antimicrobial activity of antimicrobial agent. The results were illustrated in Fig. 4 and 5.

Effects of temperature: The results suggest that the temperature did not affect the antimicrobial activity of dialysate (antimicrobial agent) as the antimicrobial activity

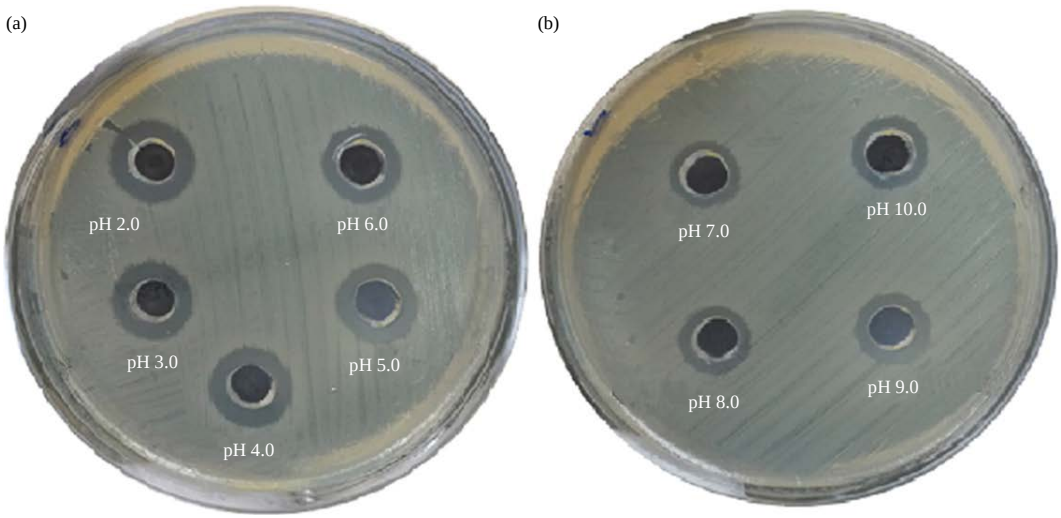

Fig. 5(a-b): Effects of pH on antimicrobial agent

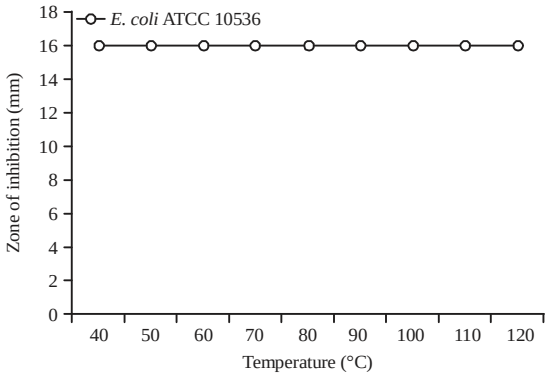

Fig. 6: Effects of temperature on antimicrobial agent

(zone of inhibition) was constant as 16 mm throughout the different temperatures. Therefore, the antimicrobial agent was highly heat stable. The results were illustrated in Fig. 6 and 7.

Determination of Minimum Inhibitory Concentration (MIC): The results suggest that the MIC value of partially purified antimicrobial agent was found to be 64 μg mL^{-1} whereas, MIC value of standard antimicrobial agent (Augmentin) was found to be 8 μg mL^{-1}.

DISCUSSION

In this study, it was found that the *Bacillus pumilus* SAFR-032 is a predominant bacterium in the soil of Unaizah, Al Qassim province of Saudi Arabia, which produces antimicrobial agent antagonistic to pathogenic bacterium *E. coli.* The results of optimization of fermentation conditions suggest that, the soybean casein digest broth and the Nutrient broth were the best and worst fermentation media, respectively, which furthermore suggest that the soil isolate does not produce antimicrobial agent in an ordinary medium and needs highly nutritious medium for the production of antimicrobial agent. The production of antimicrobial agent also affects by changing the incubation time, temperature and fermentation medium[2,4,8].

This study also suggests that the antimicrobial agent has either the peptide chain or peptide moiety in its core structure because the antimicrobial agent was successfully isolated by ammonium sulphate method as it is a well known method used to isolate the proteinaceous bioactive agents from the sample. The results of dialysis suggest that, the antimicrobial agent has molecular weight around 8000 Daltons or higher the 8000 Daltons. This antimicrobial agent is very sensitive to changes in the pH as the highest antimicrobial activity was in an acidic pH and the lowest in an alkaline pH. The antimicrobial agent is highly heat stable as there were no effects of temperatures on its activity[2,4,8]. Minimum Inhibitory Concentration (MIC) of antimicrobial agent was compared with the MIC values of standard antimicrobial agent, which suggests that the antimicrobial activity of isolated antimicrobial agent is very close to the antimicrobial activity of standard antibiotic and therefore it might be used in the future to treat the *E. coli* infections.

In previous studies, the researchers have not described the optimum growth conditions as well as optimum fermentation conditions of this soil strain. Some researchers

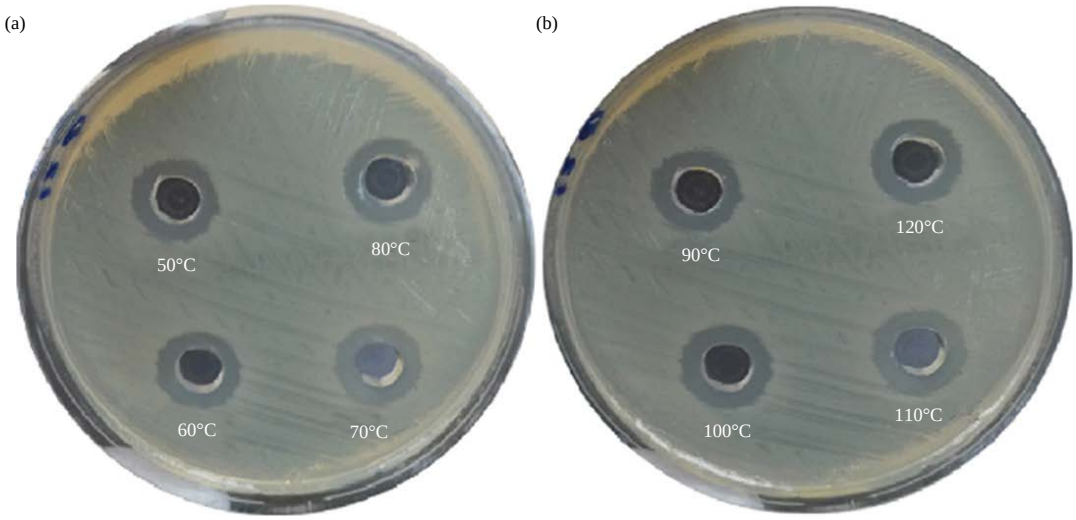

Fig. 7(a-b): Effects of temperature on antimicrobial agent

had been observed that *B. pumilus* produces substances with antibacterial activity, but very little work has been done to isolate these substances. Gilliver[30] had observed that the strains of *B. pumilus* produce antibacterial activity, but he did not attempt to isolate the active compound. Dvonch and Benedict[31] had isolated an antibacterial agent from a strain of *Bacillus pumilus*, which they showed that, it is similar to subtenolin, an antibiotic from *Bacillus subtilis*. Borowski[32] and others obtained an antibiotic, tetaine from a strain of *B. pumilus*. Bhate[33] had described that *B. pumilus* produces a noble antibiotic called pumilin but he could not do more research on this antibiotic.

In one of the recent studies, the researchers described that *Bacillus pumilus* (NKCM 8905) and *Bacillus pumilus* (AB211228) produce the antimicrobial agent against the pathogenic *E. coli* but they have not described the quantitative antimicrobial efficacy against the *E. coli* and characterization of those antimicrobial agents[34]. In one more study, the Liefert *et al.*[35] described that *Bacillus pumilus* CL45 produces antibiotic against the plant pathogens but they did not put more effort to show the antimicrobial efficacy against the *E. coli*. Thus, by comparing the results of current study with the previous studies, the researchers found that, the present study is more significant than the previous studies as there is no significant information available about the antimicrobial profile of *Bacillus pumilus* SAFR-032.

CONCLUSION

The present study is very significant for the antimicrobial drug discovery and furthermore to the healthcare industry. Therefore, it might be a great endeavor for the healthcare industry, in order to the treatment of *E. coli* infections by using this antimicrobial agent. Thus, the results of current study warrant further investigations to fully standardized and establish the antimicrobial profile of effect(s) of this soil isolate.

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