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Antibacterial Potentials of Surfactins against Multidrug Resistant Bacteria.

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ABSTRACT

The evolution of bacterial antagonist of three types of surfactin family (surfactin, lichenysin and pumilacidin) against multi-drug resistant bacteria (MDR) has been studied. The (MDR) bacteria owner of more than one antibiotic resistance genes such as *mecA*, *neo* and *cat* resistance genes. plasmids digestion using *E.CoRi*; neomycin (393bp) and chloroamephnicole (1280 bp) genes were released from plasmids pMG113 and pMG114 respectively and were transformed into *E. Coli* DH5α cells. The selective production from the strains of *B. amyloliquefaciens* S499, *B. amyloliquefaciens* FZB42 and *B. subtilis* ATCC 21332 were used to produced single (surfactin type) by 309, 1146 and 1431 $mg.L^{-1}$ respectively. While, *B. licheniformis* ATCC 14580 and *B. pumilus* were used to produce 503 and 852 $mg.L^{-1}$ of single (lichenysin and pumil acid in types) respectively. the maximal surfactins types productivity was observed at k_La values 0.085 s^{-1}. The primary antibiotic susceptibility testing was performed using ampicillin, neomycin and chloramphenicol standard antibiotics susceptibility disks. The mother strain *E.Coli* DH5α (non resistant) showed complete sensitive susceptibility patron, the modified strain *E.Coli* EMG01 (ampicillin, neomycin multidrug resistant) showed complete resistant pattern to ampicillin and more than 10 µg of neomycin. While, The modified strain *E.Coli* EMG02 (ampicillin, chloramphenicol multidrug resistant) showed complete resistant pattern to ampicillin and more than 5 µg of chloramphenicol. the diameter of the inhibition zone, (MICs) and (MBCs) were defined to determine the surfactins antibacterial activity. The data collected confirmed the bactericidal and bacteriostatic of surfactin against multidrug resistant bacteria.

Keywords: Lipopeptide, Surfactin, *Bacillus*, Multidrug resistant bacteria.

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INTRODUCTION

The global spread of microbes which have an resistance genes to antibiotic and its resistance mechanisms has been studied in detail on a global scale. Due to the increasing resistance to newer generations of antibiotics, there is higher burden on the healthcare profession to prevent the spread of these resistant pathogenic organisms [1]. Infection-related mortality rates in developing countries might return to those of the early 20th century. bacteria have been reported to show increased resistance towards common antibiotics which have been used therapeutically for the treatment of infectious diseases [2]. The evolution of multi-drug resistant pathogenic organisms, especially the types had become a important concern to health care professionals due to its almost total possess genetic resistance transfer factor (RTF) for more than one antibiotic resistance factors such as *mecA* (β-lactamase) gene have been established to determine the resistance to beta-lactam antibiotic [3] and *neo* (aminoglycoside 3'-phosphotransferase) resistance gene to aminoglycoside class of antibiotics such as neomycin [4], while the mechanisms of resistance to chloramphenicol is conferred by the *cat* (chloramphenicol acetyltransferase) gene [5]. The spread of multi-drug resistant microbs in the last years has become a massive healthcare snag. The discreet use of antibiotics and discovered of new antimicrobial agents seem to be the mutual protocol taken to combat this challenge and its remain as microbiologists mission [6]. The microorganisms control extends far beyond medical uses: The vast majority of one to two million tonnes of antibiotics manufactured today is used in the veterinary and agricultural sectors, so must limit the use of antibiotics in animal husbandry is achieved while maintaining the security and humanitarian supplies [7]. The growing antimicrobial resistance in commensal microflora, *Escherichia coli* is a usual microorganism of the aerobic flora in humans and animals pathogenes, strains of *E. coli* possess several virulence factors, the major virulence factor is the greater antimicrobial resistance susceptibility pattern, the *E. coli* genetics was revolutionizing by using integration vectors to create fusions with other genes was quickly followed [8]. The numbers has raised a persistent need for lipopeptides as alternative antimicrobial agents to be used in dairy products and food preservation as well as in clinical applications [9]. The highly demand of microbial lipopeptides is surging due to their little intoxication and ecological opportune nature composition with the enormous domains of possible applications and other utility in human welfare [10]. The lipopeptides production on large scale and application considered restricted by the high cost of their production, therefore the economical large scale production remains a challenge [11]. In the year 2003, the antibiotic daptomycin (lipopeptides biosurfactant) It has been approved in USA by Food and Drug Administration (FDA) to treat the infections of dermal pathogens belongs to the group of gram-positive bacteria [12]. The four families of *Bacillus* lipopeptides; surfactin, iturin, fengycin and kurastiken were used in biological control of plant diseases which showed antagonistic activities against various phytopathogens [13]. Other applications of lipopeptides extend beyond human health care, including use of surfactin against plant virus [14]. In general, the lipopeptides antibacterial properties is correlated with the addition of the longest length of convenient lipid part (C14-C16) of carbon atom chain and lipopeptides containing higher carbon atoms, which improve the antimicrobial activity [9, 15]. Surfactin is the main representative of the families of bio surfactant lipopeptide, which is produced for the first time from the genera of *Bacillus spp*. Surfactin is an cyclic lipoheptapeptide interlinked with peta-hydroxyfatty acid of C_{12} to C_{16} carbon atom chain length in the a cyclic lactone ring structure form [15]. The same biological activities such as surface liquid interface and active cells membrane behaviors were achevied in the bacterial strains which produce surfactins with the same amino acids sequence. This biological activities are highly importance in various processes of biotechnology and make the lipopeptide surfactin a sturdy choice for the many global purposes in industrial, pharmaceutical, agricultural and ecological protection [16]. Beside that, the levels of surfactin production and its composition of amino acids depends not only upon the nature of the producers strains but also on supply of bioprocess conditions [17]. All collected results at the various study conditions, *Bacillus amyloliquefaciens* strains produced three families of lipopeptides; surfactin, fengycin and iturin (mycosubtilin type). *Bacillus subtilis* strains produced two families of lipopeptides surfactin and fengycin (plipastatin type) or no production. While, strains of *Bacillus licheniformis* and *Bacillus pumilus* produced only surfactin family with two types of lipopeptides (lichenysin and pumilacidin, respectively) [18]. By discovering at the end of twenty century the surfactins antiviral and antimycoplasma properties leads to the suggestion of its use to guarantee the hygienic security of various pharmaceutical and biotechnological manufacture such as (antibacterial, antitumor and cholesterol lowering activities) [19]. In view of the paucity of research with regards to environmental assessment of resistant strains, different potent antimicrobial agents (surfactin, lichenysin and pumilacidin) are used the present study and its selectivity production was conducted. Also, investigated the susceptibility patron with antibiotics of the multidrug resistant *E. coli* strains was determined. While, during all lipopeptides families history, surfactin was the first studied for its potential biological and pharmaceutical applications.

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MATERIALS AND METHODS

Test microorganisms and cultivation condition

The tested bacterial strains and various plasmids used in this study are presented in (Table 1), The cultures collections of *B. subtilis subsp. subtilis* str 168 and *B. subtilis* ATCC 21332; *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* S499; *B. licheniformis* ATCC 14580 in addition to *B. pumilus* were used to produce single (surfactin) lipopeptides family production [20]. Cultures were performed in 0.085 S^{-1} of voulmetric oxygen supply co-effecient (K_La) codition (50 mL) standard Erlenmeyer flask and shaking frequency of 250 min^{-1} and the relative filling volume (*Rv*) of 0.05 $mL.mL^{-1}$ (liquid/flask), this condition was tested in each *Bacillus* strain, the cultures were performed during 48 h of fermentation (stationary phase) at 30°C in modifed Landy MOPS medium with glutamic acid, presented results are means of triplicate experiments with its standard deviation [21]. The *E. coli* strains was cultivated aerobically in Luria-Bertani [LB] medium and Mueller-Hinton [MH] agar for disc diffusion method and incubated at 37°C. The medium was used to grow *E. Coli* DH5α derivatives supplemented with 5, 20 and 50 $\mu g.mL^{-1}$ of chloramphenicol, neomycin and ampicillin antibiotics respectively, while [SOC] medium was used to grow the transformed cells.

Table 1: Strains and plasmids used in the study.

Microorganisms	Description	Reference
***E. coli* DH5α**	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^-m_K^+$), λ⁻	**Lab stock**
E. coli EMG01	Ap^R Nm^R	This study
E. coli EMG02	Ap^R Cm^R	This study
Plasmids		
pGEM -T Easy	Cloning vector, Ap^R	**Promega**
pDG1661	Ap^R Cm^R Spc^R	**Guerout[22]**
pBG180	**P_{repU}-*neo*; Ap^R Nm^R**	**Hussein[23]**
pMG113	**Ap^R Nm^R**	**This study**
pMG114	**Ap^R Cm^R**	**This study**

Cloning conditions and transformation protocol

Neomycin gene was released from pBG180 plasmid by *Xba*I and *Ban*II double digestion and then cloned in pGEM-T Easy vector with ratio from 3:1 as described from Promega, this plasmid was named pMG113, while chloroamephnicole gene was released from pDG1661 (BGSC accession: ECE112, GenBank U46196) plasmid by *Mun*I and *Sph*I double digestion and then cloned in pGEM-T Easy vector, this plasmid was named pMG114. Ligation reactions were mixed well and incubated overnight at 16ºC for the maximum number of transformants. Both plasmids pMG113 and pMG114 were introduced into *E. Coli* DH5α cells by heat shock, following the manufacturer's protocol. Transformants were selected on LB agar medium supplemented with the selective antibiotic(s); 50 $\mu g.mL^{-1}$ ampicillin, chloramphenicol 5 $\mu g.mL^{-1}$ and 20 $\mu g.mL^{-1}$ neomycin and incubated for 24 h at 37ºC. Colonies from both transformants were picked and grown 24 h at 37°C in LB medium complemented with with 5, 20 and 50 $\mu g.mL^{-1}$ of chloramphenicol, neomycin and ampicillin antibiotics respectively. Plasmids were isolated from the transformed cells using the (Gene Direx plasmid miniPREP kit, USA). Restriction analysis of the plasmids was performed using *EcoRI* (Fermentas, Germany) to check for inserts.

Table 2: (pGEM-T) Easy vector system ligation mixture

Reaction Component	Standard reaction	Positive control	Background control
2X Rapid Ligation Buffer, T4 DNA Ligase	5 µl	5 µl	5 µl
pGEM-T Easy Vector (50 ng)	1 µl	1 µl	1 µl
PCR product	X µl	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl

Nuclease-free water to a final volume of	10 µl	10 µl	10 µl

Agarose gel electrophoresis

Agarose was dissolved in 100 mL 0.5X TBE buffer pH 8.8 with 1% concentration. Gel was coloured by addition of Gel Red 10 µL/100 mL, then it was run in an electrophoresis unit using 0.5X TBE buffer pH 8.8 at 100 volts for two hours. Gel was photographed using gel documentation system for verification of resistant antibiotics insertion, the inserts were checked by plasmids digestion using *EcoRI*; neomycin (393bp) and chloroamephnicole (1280 bp) genes were released from plasmids.

Surfactin family determination

Three types of surfactin family (surfactin, lichenysin and pumilacidin) were determined by reverse phase high-performance liquid chromatography (HPLC), the produced lipopeptides were extracted after microbial batch fermentation. The bacterial cells were removed from fermented culture by centrifugation at 15.000 rpm at 5°C for 20 min. The total yield was collected before analysis by (HPLC) in C_{18} column (5 µm; 250 by 4.6 mm, VYDAC 218 TP, Hesperia, CA), the mobile phase was isocritical acetonitrile-water-trifluoroacetic acid solvent system 80:20:0.5 [vol/vol/vol] for all types. (20 µl) of collected samples were injected and then eluted at a flow rate of 1 $ml.min^{-1}$. Surfactin (Sigma), lichenysin and pumilacidin were purchased with purity of 98% as standards. The retention time and second derivatives of UV-visible spectra (Waters PDA 996 photodiode array detector; Millenium Software) of each peak were used to identify the eluted molecules [20].

Antibacterial susceptibility testing

Primary antibiotic susceptibility testing was performed using Kirby-Bauer disk diffusion method according to the protocol described by the clinical laboratory standards institute (CLSI) guidelines using following standard antibiotics discs (Bio-Rad): ampicillin, neomycin and chloramphenicol with doses of 5, 10 and 30 µg for each antibiotic susceptibility disk. The isolates were inoculated onto a Muller-Hinton agar (Oxoid) plate and antibiotic discs were placed on the surface of the agar. The plates were incubated for 24 h at 37°C. After incubation, the diameter of the inhibition zone in millimeters was measured to assess resistance or susceptibility according to the interpretation criteria for *E coli* (ATCC No. 25922) established by the (CLSI) guidelines. Multidrug resistance is defined as resistance to more antibiotics belonging to different antibiotic classes to accurately determine resistance. Isolates that were resistant to more than one antibiotic are considered multi drug resistant (MDR) as per CLSI guidelines [24].

Surfactins antibacterial testing

Antibacterial activity test was determined by using Mueller-Hinton agar plates and carried out according to the protocol described by (CLSI) guidelines as follows: The overnight bacterial cultures grown on Mueller-Hinton broth (Oxoid) were adjusted to the density of 0.5 McFarland turbidity standard unit. The inoculation of the tested bacteria were streaked on to Mueller-Hinton agar (Oxoid) plates using a sterile swab. Sterile filter discs (diameter 6 mm) (Whatman Paper No(1): 6 mm, England) were impregnated with (20 µl/disc) of purified produced surfactins types (surfactin, lichenysin and pumilacidin), the disks were placed on the appropriate agar medium, distilled water was used as negative control. After incubation at 37°C for 24 h, the diameter of the inhibition zone in millimeters was measured. The diameter of the zones of inhibition around each of the discs was taken as measure of the antimicrobial activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded with its standard devisions. While, The minimal inhibitory concentrations (MICs) $\mu g.mL^{-1}$ were defined as the lowest concentrations of surfactins that completely inhibited the growth of each *E. coli* strain. The surfactins cultures media (supernetant) were purifuied and injected within the culture medium (Mueller-Hinton broth, further progressive dilutions to obtain the final concentrations of zero to 350 $\mu g.mL^{-1}$. The tubes were inoculated with 100 colony forming unit per mL ($cfu.mL^{-1}$) and incubated at 37°C for 24 h. The growth control consisting of clear media and growth culture (negative and positive control) at the same dilutions as used in the experiments were employed. In additin, the minimum bacterial concentration (MBCs) were carried out to check whether the test *E. coli* bacterial cells were killed or only their growth was inhibited. Mueller-Hinton agar was prepared and sterilized at 37°C for 15 minutes, the medium was poured into sterile petri dishes and were allowed to cool and solidify. The contents of the MBCs in the serial dilution were then sub-cultured onto the prepared medium, incubation

was made at 37°C for 24 h, after which each plate was observed for colony growth, the lowest concentration of surfactins without a colony growth was recorded as the MBCs [24].

RESULTS AND DISCUSION

Single surfactin production

The production selectivity has been observed from multiple lipopeptides families produced by *Bacillus* strains which was very correlated with oxygen transfer condition. The strains of *B. amyloliquefaciens* S499, *B. amyloliquefaciens* FZB42 and *B. subtilis* ATCC 21332 were used to produced single (surfactin type). While, *B. licheniformis* ATCC 14580 and *B. pumilus* were used to produce single (lichenysin and pumilacidin types) respectivelly. Thus, (K_La) value of 0.085 S^{-1} at small relative filling volumes with fast shaking frequencies conditions were established. The extrusive concentrations levels of produced surfactins types (surfactin, lichenysin and pumilacidin) were observed in Table (3). The obtained production of *B. amyloliquefaciens* S499, *B. amyloliquefaciens* FZB42 and *B. subtilis* ATCC 21332 were 309, 1146 and 1431 $mg.L^{-1}$ respectivelly of single surfactin with traces of other lipopeptides. While, the production of *B. licheniformis* ATCC 14580 was 503 $mg.L^{-1}$ of single lichenysin and *B. pumilus* 852 $mg.L^{-1}$ of single pumilacidin. A similar positive correlation of k_La values on surfactin production by the same strains of *Bacillus* was reported, which was the maximal surfactins types productivity was observed at k_La values more than 0.04 s^{-1} for (surfactin, lichenysin and pumilacidin) types [20].

Table 3: The levels of surfactins productions

Bacillus strains	**Surfactin type**	**Production $mg.L^{-1}$ ± SD**
B. amyloliquefaciens FZB42	Surfactin	309 ± 13.77
B. amyloliquefaciens S499	Surfactin	1146 ± 9.46
B. subtilis ATCC 21332	Surfactin	1431 ± 9.11
B. licheniformis ATCC 14580	Lichenysin	503 ± 10.17
B. pumilus	Pumilacidin	852 ± 8.32

**** Results are means of experiments ± standard deviation (SD).***

Antibacterial susceptibility of (MDR) bacteria

In the present study the effects of primary antibiotic susceptibility testing was performed by *E.Coli* DH5α and two multidrug resistant *E.Coli* strains (EMG01 and EMG02). The effect of three antibiotics which widely involved in the resistant mechanisms were examined. Ampecilin was used for β-lactamase resistant gene involved bacteria [3], neomycin was used for aminoglycoside 3'-phosphotransferase resistant gene [4] and chloramphenicol acetyltransferase gene [5] have been established to determine the resistance to chloramphenicol. The antibiotic susceptibility of (MDR) *E. coli* strains with its inhibition zones diameters Table (4).

Table 4: Antibiotic susceptibility of multidrug resistant bacteria

Antibiotics		***E. coli*** **inhibition zone mm ± SD**		
Type	**Conc**	***E. coli*** **DH5α**	***E. coli*** **EMG01**	***E. coli*** **EMG02**
Ampicillin	5 µg	17.6 ± 0.2	R	R
	10 µg	19.9 ± 0.4	R	R
	30 µg	23.6 ± 0.7	R	R
Neomycin	5 µg	19.2 ± 0.8	R	19.1 ± 0.5
	10 µg	21.5 ± 0.3	R	21.1 ± 0.6
	30 µg	27.9 ± 0.6	13.8 ± 0.2	27.4 ± 0.8
Chloramphenicol	5 µg	19.7 ± 0.3	19.6 ± 0.4	R

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	10 µg	23.2 ± 0.5	22.8 ± 0.6	12.8 ± 0.2
	30 µg	29.6 ± 0.3	29.2 ± 0.7	20.3 ± 0.3

R: resistant (inhibition zone was 10 mm or less).

The mother strain *E.Coli* DH5α showed complete sensitive susceptibility patron with all concentrations of antibiotics used. The modified strain *E.Coli* EMG01 showed complete resistant pattern to ampicillin and more than 10 µg of neomycin. While, the modified strain *E.Coli* EMG02 showed complete resistant pattern to ampicillin and more than 5 µg of chloramphenicol. The multidrug resistance is defined as resistance to more than one antibiotic belonging to different antibiotic mechanisms to accurately determine resistance. The modified strain *E.Coli* EMG01, EMG02 were resistant to more than one antibiotic so considered multidrug resistant strains according to (CLSI) guidelines [24].

Surfactins antibacterial potentials

The effects of different produced surfactins types against *E.Coli* DH5α (non resistant), *E.Coli* EMG01 (ampicillin, neomycin multidrug resistant) and *E.Coli* EMG02 (ampicillin, chloramphenicol multidrug resistant) were evaluated as measured inhibition zones. The minimal inhibitory concentrations (MICs) and the Minimum bacterial concentration (MBCs) $\mu g.mL^{-1}$ of the net concentrations of surfactins types (surfactin, lichenysin and pumilacidin) were tested and summarized in table (5).

Table 5: Antibacterial activity of surfactins against MDR bacteria

Treatments	*E. coli* DH5α			*E. coli* EMG01			*E. coli* EMG02		
	Inh Zone mm ± SD	MICs $\mu g.mL^{-1}$	MBCs $\mu g.mL^{-1}$	Inh Zone mm ± SD	MICs $\mu g.mL^{-1}$	MBCs $\mu g.mL^{-1}$	Inh Zone $\mu g.mL^{-1}$	MICs $\mu g.mL^{-1}$	MBCs $\mu g.mL^{-1}$
B. licheniformis **(Lichenysin)**	10.8 ± 0.4	280	310	10.2 ± 0.7	280	310	10.9 ± 0.6	280	310
B. pumilus **(Pumilacidin)**	13.3 ± 0.6	300	320	13.1 ± 0.3	300	320	13.7 ± 0.8	300	320
B. amylo **FZB42 (Surfactin)**	11.4 ± 0.9	290	300	11.2 ± 0.7	290	300	11.8 ± 0.2	290	300
B. amylo **S499 (Surfactin)**	16.4 ± 0.7	320	350	16.3 ± 0.2	320	350	16.8 ± 0.6	320	350
B. subtilis **21332 (Surfactin)**	19.1 ± 0.5	340	370	18.9 ± 0.4	340	370	18.2 ± 0.7	340	370

**** Results are means of experiments ± standard devision (SD).***

The treated with 20 µl of various surfactins types notably inhibited all test *E.Coli* growth, the strong inhibition zone was 19.1 mm for the surfactin produced by *B. subtilis* ATCC 21332, which also inhibit the growth of multidrug resistant *E.Coli* EMG01 and EMG02 strains by inhibition zones of 18.9 and 18.2 mm respectively with relatively inhibitory concentrations 340 and 370 $\mu g.mL^{-1}$ of (MICs) and (MBCs) for all strains. The treated with surfactin produced from *B. amyloliquefaciens* S499 given 16.4, 16.3 and 16.8 mm for *E.Coli* DH5α, EMG01 and EMG02 respectively with 320 and 350 $\mu g.mL^{-1}$ of (MICs) and (MBCs). The surfactin produced from *B. amyloliquefaciens* FZB42 given 11.4, 11.2 and 11.8 mm with 290 and 300 $\mu g.mL^{-1}$ of (MICs) and (MBCs). While, the pumilacidin produced from *B. pumilus* given 13.3, 13.1 and 13.7 mm respectively with 300 and 320 $\mu g.mL^{-1}$ of (MICs) and (MBCs). In addition to the lichenysin produced from *B. licheniformis* given 10.8, 10.2 and 10.9 mm of inhibition zones respectively with 280 and 310 $\mu g.mL^{-1}$ of (MICs) and (MBCs) for all strains. Based on the latest assays confirmed the bactericidal and bacteriostatic of surfactin especially against multidrug resistant bacteria. Also, the same promising antibacterial behaviour of surfactin types was observed towards all *E. coli* strains. But, it is clear that, a positive correlation between surfactin production levels and maximum

inhibition zone were determined. The probable reason for the lowest (MICs) and (MBCs) reported due to the production force of the *Bacillus* strain [13]. The efficiency of surfactins is moderated by comparison of antibiotics especially in low concentration but without chance to produce drug resistant strain of surfactin. Consequently, the occurrence potential of surfactin drug resistant strains is very low due to its specific natural composition that might function as a barrier to bioactive compounds that are reason for antibacterial activity [15, 25].

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