Isolation and Screening of Biosurfactant-Producing Bacteria from Hydrocarbon-Contaminated Soils in Awka, Southeast, Nigeria

ABSTRACT

Biosurfactants are a variety of groups of active compounds produced by various types of microorganisms. Bacterial surfactants are suitable for a range of applications in the oil industry, food, agriculture, pharmaceutical, cosmetics and bioremediation.

Aim: The present study aimed to isolate and screen bacterial species for biosurfactant production.

Methodology: Two hundred soil samples collected from hydrocarbon contaminated soils in Awka, Nigeria, were cultured on casein starch agar. The isolates were screened for biosurfactant production stability and antimicrobial activity.

Results: Primary screening on the 87 recovered isolates using drop collapse, oil spreading, haemolysis tests showed that 16 isolates (18.39%) were biosurfactant producers. Secondary screening resulted in 9 isolates with good emulsification index (E24) ranging from 42.18% to 74.07%. The culture supernatant of the isolates containing biosurfactants were stable at varying pH (2 – 12), temperature (4 - 121˚C) and salinity (2 – 20%). Most isolates have antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. Based on oil spreading, emulsification index and antimicrobial activity, four of the isolates, ASP, AS1, DS4, DS8, were selected as most efficient in biosurfactant production. Phylogenetic analysis based on 16S rRNA genes classified ASP as *Streptomyces* sp, AS1 as *Actinomyces* sp, DS4 and DS8 as species of *Alcaligenes faecalis.*

Conclusion: All the species investigated except *Actinomyces* are known to belong to biosurfactant producing bacteria. *Actinomyces* sp AS1, is likely to be a strain representing a new class of biosurfactant producers.

Key words : Actinomyces, Biosurfactant, Emulsification Index, oil spreading, Antimicrobial

1. INTRODUCTION

Surfactants are a class of chemical compounds commonly obtained by chemical synthesis from petroleum derivatives and have been widely used in various industrial sectors (petrochemical, pharmaceutical, food, agro-chemical, and hygiene/cosmetics) as detergents, emulsifiers, adhesives, flocculants, foam agents, demulsifiers and penetrants [1].

Search for sustainable alternatives to production of chemical using renewable substrates and natural processes are important to replace existing surfactants [2, 3].

The search for microorganisms that produce compounds of economic interest, including surfactants has driven biotechnology in recent decades [3]. Microbial surfactant or biosurfactants are surface active compounds synthesized by fungi, yeast and bacteria. However, due to their great metabolic versatility, bacteria are the most traditional and well-known microbial surfactants [3].

A screening study showed that ability to synthesize biosurfactant is regularly present in microbial populations of soil and sediments from Polar Regions [4], once they are involved in the uptake and solubilization of hydrocarbon [5].

The biosurfactants have attracted attention because of their low toxicity, biodegradability, and ecological acceptability. Also low cost raw materials such as agricultural and industrial waste can be used for their production [6].

Industrial applications of biosurfactants are many. Environmental use involves biodegradation and bioremediation of pollutants, while in the food industry it plays an essential role in food consistency and textures [3,7]. In agriculture biosurfactants promote plant growth through their antimicrobial activity and increase the interaction between plants and beneficial microorganisms [8].

It can also enhance the degradation of chemical pesticides found in agricultural soil. The antimicrobial, antitumoral, antiadhesive, antibiofilm, anti-inflammatory, and immune-suppressive properties demonstrated by biosurfactant is widely explored in therapeutic applications [9, 10, 11].

There are several techniques that when used together, can be efficient in the prospecting of microorganisms producing surfactant compounds. Among the most employed are the emulsification index, droplet collapse and surface tension measurement [12].

Regardless of significant advances in this field, the biosurfactant-producing potential remains scarce or unknown for most bacterial species. Factors such as habitat, physiological, genetic and biochemical characteristics of bacteria may influence the production, composition as well as physiological properties of biosurfactants [13, 14, 15].

Considering the importance of biosurfactants produced by microorganisms, this research work was conducted to screen for biosurfactant-producing bacterial isolates recovered from hydrocarbon contaminated soil in Awka Town, Southeast Nigeria.

2. MATERIALS AND METHODS

2.1 Sample collection

A total of 200 samples were randomly collected from hydrocarbon-contaminated soils, which include the abbatoirs, mechanic workshops and refuse (waste) dumps in Awka locality (Lat. $6^{o}12'36"$N, Long.$7^{o}4'27"$E), Southeast Nigeria. The samples were collected in sterile polythene bags, at a depth of 5 - 10 cm of the soil, and taken to Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Laboratory for analysis.

2.2 Isolation of bacteria

The bacterial organisms from the soil samples were isolated following the modified methods described by [16] and [17] on starch casein agar medium. Starch Casein agar (SCA) composition - soluble starch, 100g; casein, 0.3g; KNO_3, 2.0g; NaCl, 2.0g; K_2HPO_4, 2.0g; $MgSO_4$. $7H_2O$, 0.05g; $CaCO_3$, 0.02g; $FeSO_4.7H_2O$, 0.1g; Agar, 20.0g; distilled water, 1000 ml; pH 7.0. One gram of the soil sample was serially diluted ten-fold and 0.1ml of 10^{-5} and 10^{-6} dilutions spread inoculated on starch casein agar plates. The plates were incubated for 7 – 10 days at 30^0C, and colonies which showed morphological differences were isolated, purified by streak plate technique and maintained for further studies.

2.3 Screening for biosurfactant production

2.3.1 Preparation of broth culture

The pure isolate suspended in 2 ml of sterile distilled water was transferred in 50 ml of Kim's medium (g/L: $NaNO_3$, 1; $KHPO_4$, 0.1; $MgSO_4$. $7H_2O$, 0.1; CaCl, 0.1; yeast extract, 0.2; n-Hexadecane, 30ml/L; pH 6) with 3% olive oil as the sole carbon source in a 250 ml Erlenmeyer flask [18]. The flask was incubated on a rotary shaker for 7 days at 28^0C, and the content centrifuged at 10,000 X g for 15 mins. The cell free supernatant was used for further studies.

2.3.2 Crude biosurfactant extraction

The cell-free broth obtained after centrifugation was subjected to acidification using 1N HCl until reaching pH 2.0 and was left overnight at 4^0C for precipitation. The biosurfactant was extracted by treating equal volume of the sample with chloroform: methanol (2:1 v/v). The separated solvent layer was removed and the upper aqueous phase re-extracted three times as before. The combined extracts were concentrated using a rotary vacuum evaporator (Rotary vacuum evaporator RE 100, Bibby Co., UK) to give the crude extract at 40^0C [19, 20].

2.3.3 Biosurfactant Activity

Hemolytic activity: Haemolysis was carried out on plates of blood agar medium supplemented with 5% human blood. The culture supernatant of the isolates was inoculated, and the blood agar plates incubated at 28^0C for 7 days. The plates were then examined for zone of clearance around the colonies [21].

Drop collapsing test: Biosurfactant production by the isolate was screened using the qualitative drop-collapse test described by [22]. Olive oil (2 µL) was added to 96-well microlitre plate. The plate was equilibrated for 1 hr at 37^0C and 5 µL of the culture supernatant of the isolate added to the surface of the oil in the well. The shape of drop on the oil surface was observed after 1 min. If the drops collapsed, the result indicates positive but if the drops remain intact, the result is negative. Distilled water was used as a negative control.

Oil spreading assay: The oil-displacement method was used to detect the activity of biosurfactant [23]. For the assay, 10µL of crude oil was added to the surface of the 40 ml of distilled water in a petri dish, to

form a thin oil layer. Then, 10µL of the cell-free supernatant of the isolate was gently placed on the centre of the oil layer. If the biosurfactant is present in the supernatant, the oil is displaced and a clearing zone formed. The diameter of the cleared zone on the oil surface correlates to surfactant activity.

2.3.4 Preliminary identification of biosurfactant

Phenol-H_2SO_4 test: To 1 ml of cell-free supernatant was added 1 ml of 5% phenol. To this mixture was added 5 ml of concentrated H_2SO_4 drop by drop, until orange color was developed. The orange color indicates the presence of glycolipids containing biosurfactant [24].

Biuret test: This test was used to detect the presence of lipopeptide-type of biosurfactant [25]. 2 ml of crude extract solution of the isolate was first heated at 70^0C before mixing with 1 ml of 1M NaOH solution. Drops of 1% $CuSO_4$ were added slowly until violet or pink ring was observed, indicating presence of lipopeptide biosurfactant.

Phosphate test: To detect the presence of phospholipid biosurfactants, the phosphate test was conducted [26]. Ten drops of 6M HNO_3 were added to 2 ml of crude extract solution and heated at 70^0C. Then drops of 5% (w/v) ammonium molybdate were slowly added until the formation of yellow color and a fine yellow precipitate.

CTAB/Methylene blue agar: Blue agar (15g/L) composing of mineral salts-agar medium supplemented with 2% carbon sources, 0.2g/L N-cetyl N,N,N-trimethylammonium bromide (CTAB), 0.005g/L methylene blue were used to detect extracellular glycolipid producton. The presence of biosurfactants was detected by observing the development of dark blue halo around the colonies [27, 28].

2.3.5 Evaluation of biosurfactants

Evaluation of biosurfactants produced by the isolates was carried out by measuring the stabilization of the emulsion.

Emulsification index: The emulsifying capacity was evaluated by emulsification index (e24) [29]. The E24 of the isolates was determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in centrifuge tube, vortexed at high speed for 2 min and allowed to stand for 24 h. The E24 index is given as percentage of the height of emulsified layer (cm) divided by the total height of the liquid column (cm).

2.4 Stability of Biosurfactants

The effects of temperature, pH and sodium chloride concentrations on the biosurfactant produced by the isolates were determined [30,31].

Stability studies were done using the cell-free supernatant obtained by centrifuging the broth culture of the isolate at 10,000 X g for 15 mins. Four milliliter of the cell-free supernatant was heated at 50, 70, 100 and 121^0C for 1 h, cooled to room temperature and the emulsification activity measured. The

emulsification capacity of the cell-free supernatant was also determined after exposure to lower temperature (5^0C)

To study the pH stability of the cell-free supernatant, the pH was adjusted to different pH values (2 – 120 and the emulsification activity measured. The culture liquid pH was adjusted with 6.0M NaOH or HCl.

The effects of NaCl concentration (2 – 20%) on the emulsification capacity of the cell-free supernatant was also determined. The cell-free supernatant of the isolate was mixed evenly with the specific concentrations of NaCl and the emulsification index recorded.

2.5 Antimicrobial Activity of the biosurfactant

The antimicrobial activity of the biosurfactant produced by the isolate against *Escherichia coli* and *Staphylococcus aureus* was determined by agar diffusion method [32]. Nutrient agar plates seeded with 0.2 ml (1.1 X 10^6 cells/ml) of overnight test organism suspension and agar wells (6 X 4 mm) prepared on the plates by scooping out the medium with sterile cork borer. The cell-free supernatant of the broth culture of the isolate was then administered to the wells and incubated at 37^0C for 24 h. Antimicrobial activity was determined by measuring the inhibition zone diameter (in mm) around the well after incubation. Triplicate plates were used.

2.6 Spot inoculation of isolate on Agar medium

The antimicrobial activity of the isolate was also studied by spot inoculation technique on agar medium as described by [32]. The isolate was spot inoculated on SCA, the petri plate incubated at 28^0C for 7 days and then inverted for 40 min over chloroform in fumehood. Colonies of the isolate were then covered with thin layer (0.6% agar) of nutrient agar previously seeded with bacterial suspension of 1.1 X 10^6 CFU/ml and the plate incubated at 37^0C for 24 h. The zone of inhibition of growth of the test microorganism around the isolate was observed after incubation. Triplicate plates were used.

2.7 Molecular Identification of isolates

Genomic DNA (16S rRNA) was extracted using ZR bacterial DNA miniprep of Zymo Research. The DNA was PCR amplified using Taq 2X Master Mix from New England Biolabs (M0270). The forward and reverse primer used were (27F: AGAGTTTGATCMTGGCTCAG) and reverse (1525R: AAGGAGGTGWTCCARCCGCA). The cycling conditions for the amplification of the 16S rRNA were - Initial denaturation at 94˚C for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 56˚C for 30secs and elongation at 72˚C for 45sec. Followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10 ˚C. The DNA was ran on agarose gel for electrophoresis and visualized under UV transilluminator. The amplified fragments were sequenced with a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual and the sequencing kit used was BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA X were used for the genetic analysis. The evolutionary history was inferred using the Neighbor-joining method [33], and the evolutionary distances were computed using the maximum Composite Likelihood method [34].

3. RESULTS AND DISCUSSION

A total of 87 bacterial isolates were recovered from the hydrocarbon contaminated soil samples in Awka, Southeast, Nigeria, and were screened for biosurfactant production.

Primary screening using drop collapse, oil spreading, haemolysis tests showed 16 isolates (18.39%) having biosurfactant activity, while secondary screening resulted in 9 of the isolates showing good emulsification activity (Table 1). All the 9 isolates showed emulsification (E24) ranging from 42.18 to 74.07%. Based on the Emulsification index, the 9 isolates were selected for further studies.

Bacteria capable of synthesizing biosurfactants are spread over a wide habitat and can be found in various soil samples and sediments, from Polar Regions, once they are involved in the uptake and solubilization of hydrocarbons [4, 5]. It is however, known that different ecological environments, with the surrounding physical, chemical and biological conditions often play a role in the discovery of new types of bacteria and metabolites [35].

Table 1: Screening of Biosurfactants Produced by the Isolates

Isolate code	Drop collapse	Haemolysis test	Oil spreading test (Diameter of cleared zone [mm])	Emulsification Index (%)
MS 2	-	+++	-	22.73
ASF	++	-	6.5	74.07
MS3	+	-	4.0	46.88
VS9	+	++	4.9	42.18
AS28	-	+	-	19.35
AS2	-	-	-	35.71
AS1	+++	-	7.0	60.61
DS8	++	-	5.3	61.29
DS6	-	-	-	28.57
DS4	++	-	5.3	66.67
VS10	-	-	-	26.47
DS1	+	++	4.3	50.00
AS1A	-	+++	-	27.59
DS7A	+	-	4.8	51.72
VSB	-	-	-	24.00
DS7	+	-	5.7	44.28

Biosurfactants are classified based on their chemical structures that include glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids [36]. The partial characterization of the isolates is a shown in Table 2. While some of the isolates (MS3, AS1, DS4) belong to the phospholipid group, the other isolates (ASF, VS9, DS8, DS1, DS 7A, DS 7) are of the glycolipid group.

As suggested by [36], the biochemical composition of biosurfactant probably depends on the substrates utilized in the culture medium. It has been reported that the biosurfactant isolated from *Candida*

lipolytica UCP0988 showed 50.0% of the proteins, 8.0% of carbohydrates and 20.0% of lipids in the presence of refinery soybean oil [31]. Other results showed that the biosurfactant of *Lactobacillus delbruckii* produced from peanut oil cake, has a composition of 30.0% carbohydrates and 70.05 lipids [37], while a 15.0% carbohydrate and 70.0% lipid was obtained for the biosurfactant of *Candida sphaerica* UCP0995 produced from industrial waste [38].

Table 2: Characterization of the Biosurfactants Produced by the Isolates

Isolate Code	Phosphate test	Biuret Test	Phenol-H_2SO_4	CTAB/Methylene Blue Agar Test
ASF	-	-	+	+++
MS3	+	-	-	-
VS9	-	-	+	+
AS1	+	-	_	-
DS8	-	-	+	++
DS4	+	-	-	-
DS1	-	-	+	+
DS7A	-	-	+	+
DS7	-	-	+	+

Table 3, shows the effect of temperature on the biosurfactants produced by the isolates. All the isolates with few exceptions that showed decrease in biosurfactant activity at 4^0C, produced biosurfactants that have thermal stability. Similar behaviors regarding stability were also observed for biosurfactants produced by *Bacillus subtilis* and by *Nocardia* sp L-417 [39, 40].

Table 3: Effects of Temperature on Biosurfactants Produced by the Isolates

	Temperature (^{0}C)				
Isolate Code	**5**	**50**	**70**	**100**	**121**
ASF	41.38	48.28	42.42	43.33	45.16
MS3	28.13	30.00	35.71	36.13	33.35
VS9	32.31	32.46	33.52	33.70	30.57
AS1	40.63	51.52	54.83	60.00	58.06
DS8	16.67	37.86	28.33	44.83	47.06
DS4	37.93	36.36	34.48	33.33	32.26
DS1	20.34	31.23	33.58	35.16	34.25
DS7A	24.24	31.18	32.07	32.26	30.00
DS7	17.24	28.16	31.07	32.35	33.29

The influence of pH on biosurfactant produced by the isolates is shown in Table 4. The biosurfactants have good surface activity in both acidic and basic media, indicating that the variation in pH of the biosurfactant had no appreciable effect on their activities. A similar stability pattern in the environmental conditions of pH (2 – 12) was observed in the biosurfactant produced by *Candida lipolytica* UCP0988 [31].

Table 4: Effects of pH on Biosurfactants Produced by the Isolates

	pH					
Isolate Code	**2**	**4**	**6**	**8**	**10**	**12**
ASF	51.61	50.00	50.00	53.13	50.00	50.00
MS3	49.57	49.23	50.43	51.14	51.28	51.32
VS9	59.46	57.23	55.65	54.00	54.11	54.17
AS1	56.67	56.67	53.33	53.33	54.84	56.25
DS8	59.26	57.14	56.67	50.00	38.71	34.38
DS4	60.61	50.00	50.00	50.00	55.17	56.67
DS1	46.88	48.57	50.00	50.00	50.00	50.00
DS7A	46.67	50.27	50.48	51.14	52.06	53.85
DS7	55.72	55.14	52.78	52.15	52.26	52.34

Table 5 illustrates the effects of NaCl on the biosurfactants produced by the isolates. Although little changes occurred in the influence of NaCl on the biosurfactant activity of the isolates, their stability was maintained at 2 – 20% concentrations. Biosurfactants produced by bacteria with similar effects have been reported [30].

Table 5: Effects of NaCl on Biosurfactants Produced by the Isolates

	NaCl (%)				
Isolate Code	**2**	**5**	**10**	**15**	**20**
ASF	44.11	50.00	50.00	54.28	55.17
MS3	50.85	50.64	47.59	44.24	45.48
VS9	47.27	42.85	47.78	47.69	47.34
AS1	56.25	51.85	55.88	54.54	40.00
DS8	41.13	42.58	43.33	46.67	48.48
DS4	54.55	57.58	57.14	52.94	52.63
DS1	50.00	50.00	50.30	50.45	50.27
DS7A	48.57	50.76	51.25	53.33	53.66
DS7	5036	50.33	47.59	46.18	44.24

The antimicrobial activity of the biosurfactants produced by the isolates on *Escherichia coli* and *Staphylococcus aureus* is as presented in Tables 6a and 6b. Two of the isolates showed biosurfactant activity against gram negative *E. coli* while the other six had activity against gram positive *Staph aureus* (Table 6a). On the spot inoculation (Table 6b), two of the isolates showed activity against both gram positive and gram negative test organisms while five others had no activity on either of the test organisms.

The variation in the activity of the biosurfactants produced by the isolates and that of spot inoculation may have resulted from the media and procedure used for the study.

The biological activity demonstrated by biosurfactants has been widely demonstrated and extensively reported [41, 42, 43]. Although the mechanism is not fully elucidated, the antimicrobial activity of

biosurfactant is attributed to their disturbance in the integrity of cytoplasmic membrane, leading to the formation of pores and ion channels, increased permeability, metabolite leakage and cell death [44].

Characterization of the isolates – Based on the values of drop collapse test, oil spreading technique and emulsification index, isolates ASF, AS1, DS8 and DS4 were observed to be the most efficient biosurfactant producers, and were selected for further investigations.

Table 6a: Antimicrobial Activity of the Biosurfactant Produced by the Isolates on *Escherichia coli* and *Staphylococcus aureus*

	Zone of Inhibition (mm)	
Isolate Code	***Escherichia coli***	***Staphylococcus aureus***
ASF	25	-
MS3	-	0.8
VS9	-	15
AS1	-	49
DS8	30	-
DS4	-	14
DS1	-	18
DS7A	-	-
DS7	-	10

Table 6b: Antimicrobial Activity of Spot Inoculation of the Isolates on *Escherichia coli* and *Staphylococcus aureus*

	Zone of Inhibition (mm)	
Isolate Code	***Escherichia coli***	***Staphylococcus aureus***
ASF	-	-
MS3	-	-
VS9	6	6
AS1	3	-
DS8	-	-
DS4	-	-
DS1	10	5.6
DS7A	-	-
DS7	-	5

Phylogenetic analysis (Figure 1) based on 16S rRNA gene identified ASF as *Streptomyces* sp having 84.38% pairwise similarity with *Streptomyces carpaticus* strain N29 (NCBI accession number HQ132783) and AS1 as *Actinomyces* sp having 98.07% pairwise similarity with *Actinomyces viscosus* strain AICC 15987 (NCBI accession number MH173790). DS4 and DS8 were identified as *Alcaligenes* species having 93.0% pairwise similarity with *Alcaligenes faecalis* strain IISR-EPN BC 8 (NCBI accession number MH249778) and 98.36% pairwise similarity with *Alcaligenes faecalis* strain SD5 (NCBI accession number JQ993101) respectively.

All the isolates recovered except *Actinomyces* AS1 have been reported as biosurfactant-producing bacteria [3, 17, 36, 45, 46]. However, *Actinomyces israelii* and *Actinomyces viscosus* have been reported to produce peroxidases, extracellular enzymes used by crude oil degrading microbes [47]. Since *Actinomyces* AS1 produces biosurfactant, it is likely to be a strain representing a new class of biosurfactant producers. It can also be a potential candidate for the production of phospholipid biosurfactant that can be used in a variety of industrial application particularly for bioremediation in the oil field.

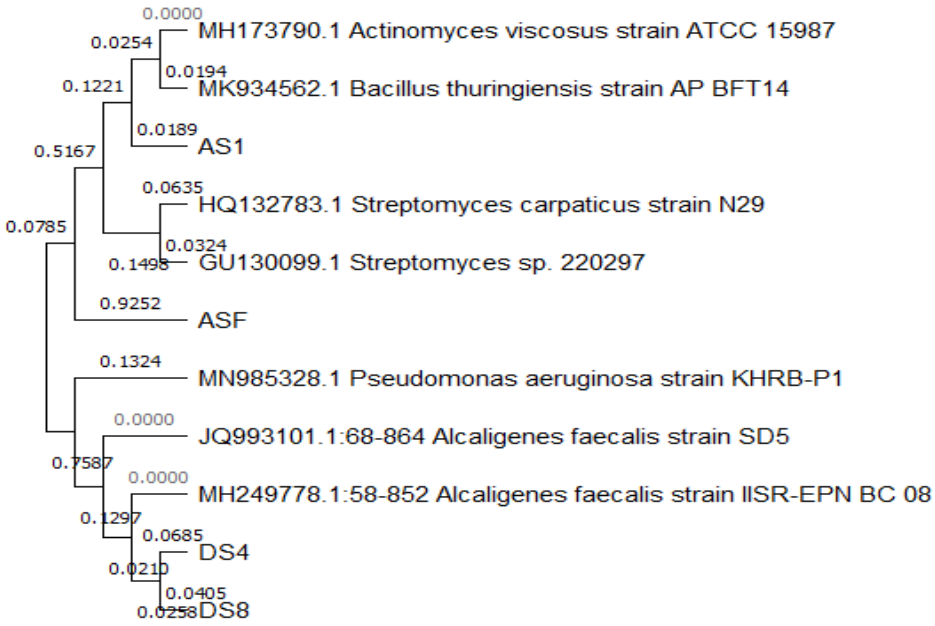

Figure 1: Phylogenetic tree showing evolutionary relatedness of the isolates.

4.0 CONCLUSION

The four isolates recovered from hydrocarbon contaminated soil were found to be active biosurfactant producers. Their biosurfactants were found to be stable at varying pH (2 – 12), temperature (4 – 121^{o}C) and salinity (2 – 20%). They were also observed to have antimicrobial activity. The isolates belong to the genera *Streptomyces*, *Actinomyces* and *Alcaligenes.*

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