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Original Article

Purification and Properties of Thermostable Fucoidanase Produced by Recently Isolated Terrestrial *Aspergillus flavus* FS018

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HIGHLIGHTS

- Fucoidanase from *Aspergillus flavus* FS018 was purified and characterized.
- Molecular weight of the enzyme was estimated to be 70kDa.
- Enzyme was active towards fucoidan consisting of α-1→4 and α-1→3 glycoside bonds in the main chains and also galactofucans group.

ABSTRACT

In this study fucoidanase produced by terrestrial *Apsergillus flavus* FS018 was purified and characterized. The pure fucoidanase enzyme was found to have an optimum activity of 20.8U/mL at 55 ºC and optimum activity of 17.2U/mL at pH 5.0. Furthermore, the fucoidanase retained 96% of its activity after 8 hours of incubation at 55 ºC. Metal ions such Mg^{2+} and Ca^{2+} ions were found to slightly enhance the activity of this enzyme while Na^{+}, K^{+} had inhibitory effect on the activity. The enzyme was found to be active towards fucoidan consisting of α-1→4 and α-1→3 glycoside bonds in the main chains and also galactofucans group. Estimation of the kinetic parameters of the enzyme revealed that K_m and V_{max} to be 1.9 mM and 0.38 mg/min, respectively when fucoidan from *Sargassum vulgare* was used as substrate. SDS-PAGE analysis of the purified enzyme revealed that it's a monomeric enzyme molecule with an estimated molecular weight of 70 kDa.

Introduction

Fucoidanases (E.C. 3. 2. 1. 44) are a group of hydrolytic enzymes which are capable of hydrolyzing the complex fucoidans polymer (sulfated and sometimes acetylated polysaccharides synthesized by brown seaweeds) to Low Molecular Weight Fucoidan (LMWF) without removal of its side substitute groups (Gurpilhares et al., 2016). This hydrolysis is carried out either by the cleavage of the glycosic bonds in the core of the fucoidan polysaccharides (endo-acting) resulting in a high reduction of its molecular weight or the cleavage of the fucoidan polymer at the edge resulting in a little decrease in its molecular weight (exo-acting) (Wang et al., 2007; Kusaykin et al., 2016). Although fucoidan can be hydrolyzed by chemical such as acids and bases, enzymatic hydrolysis preserves the sulfate functional groups on fucoidans which are reported as the major functional groups responsible for the various biological activities of sulfated polysaccharides (SPS).

Fucoidanases have recently gained attention of researchers due to the potential biological application of the LMWF (synthesized by fucoidan hydrolysis) polymer as antitumor, anticoagulant (Mourao et al.,

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2004; Li et al., 2008), anticancer (Cho et al., 2011), immunomodulatory agent (Li et al., 2008), and antioxidant (Wang et al., 2007). It has effective role in cholesterol regulation and shows antiviral activity (including the HIV) where the low molecular weight compound generated is proposed to inhibit the HIV virus by acting as competitive inhibitor between reverse transcriptase and the nucleic acid substrate (Schaeffer et al., 2000). Furthermore, low molecular weight compound produced by the hydrolysis of fucoidan has also been reported to have anti-obesity as well as renoprotective effect (Shiwei et al., 2017).

Although fucoidanases have been isolated and purified from different microorganisms and marine invertebrates (Sakai et al., 2002; Rodríguez-Jasso et al., 2008; Chang et al., 2010; Rodríguez-Jasso et al., 2010; Silchenko et al., 2013; Shvetsova et al., 2014), the industrial application of fucoidanase is still greatly limited because technologically valuable sources of these enzymes have not been found yet and the search for microorganisms with high fucoidan hydrolase activity remains a challenge. Furthermore, information on terrestrial microorganisms acting over this sulfated-polysaccharide is scarce. Hence, there is a need to search for new fucoidanase with high catalytic activities from various organisms so as to provide not only insight into the relationships between the structures and the biological activities of fucoidans, but also to improve technologies for the production of industrially important bioactive fuco-oligosaccharides. In this paper we report the properties of fucoidanase from *Aspergillus flavus* FS018, which was isolated from soil of Agricultural farm land within the University of Ibadan, Ibadan, Nigeria. To the best of our knowledge, this is one of the few reports documenting the properties of fucoidanase from terrestrial filamentous fungi and fucoidanase reported by this organism is among the highest reported in literature.

Materials and Methods

Microorganism and culture condition

The *Aspergillus flavus* 018 (GenBank Accession number MN871534.1) used in this study was obtained from the Culture Collection of the Department of Microbiology, University of Ibadan. It was recently isolated from soils of Agricultural farmland within the University of Ibadan. The organism was maintained on Fucoidan Urea Agar (FUA) medium containing (g/L): *Sargassum vulgare* fucoidan (5.0), Urea, (2.0), and agar (10.0), dissolved in 100 mM acetate buffer of pH 5.0 as described by Rodríguez-Jasso *et al*. (Rodríguez-Jasso et al., 2010).

Sample collection and preparation

Brown algae samples (*Sargassum* vulgare) used as source of fucoidan in this study were collected from Tarkwa Bay (6°24'10''North, 3°23'39''East) Beach in Lagos state, Southwest, Nigeria with the aid of National Institute of Oceanography and Marine Research (NIOMR), Victoria Island, Lagos. The samples were rinsed with several changes of distilled water to remove the adhering sand particles and then dried in an oven (Memmert oven, Type: UNB 200) at 65 °C. The dried *Sargassum* plant was further milled using a blender (Saisho Blender S-999) to obtain a fine powder and Fucoidan was extracted from the dried *Sargassum* sp using acid as described by Li *et al*. (2008) and herein referred to *Sargassum vulgare* fucoidan (SVF).

Solid state fermentation

Solid State Fermentation (SSF) was carried out in Erlenmeyer's flasks containing 5.0g of the sterile *Sargassum vulgare* powder inoculated with 1mL spore suspension (containing 4.0 x 10^7 spores/mL) and moisture content of the solid substrate adjusted to 70% (V/W) by the appropriate addition of acidified mineral solution containing gL^{-1} K_2HPO_4 (1g), $MgSO_4.7H_2O$ (0.5g), KCl (0.5g), and $FeSO_4$ (0.01g). Fermentation was carried out for 7 days with incubation at 25 ºC. After incubation, 50mL of 0.2M sodium acetate buffer (pH 5.5) was added to the fermented matter and incubated at 25 ºC with constant agitation at 150rpm for 30 minutes. Thereafter, the suspension was centrifuged at 15,000 x g for 20 minutes and the supernatant was taken as the crude fucoidanase.

Fucoidanase assay

Fucoidasnase activity was determined by measuring the amount of reducing sugars produced (Miller, 1959) from fucoidan through the following reaction: 0.9 mL of substrate (1% fucoidan from *Sargassum vulgare* dissolved with 0.2M acetate buffer, pH 4.5) was mixed with 0.1 mL of enzyme extract, and the mixture was incubated at 50 °C for 10 min (Manivasagan and Oh, 2015). One unit (U) of fucoidanase activity was defined as the amount of enzyme able to release 1 µmol of reducing sugars per minute under the assay conditions.

Fucoidanase purification

Fucoidanase purification was done by ammonium sulfate precipitation followed by dialysis and gel chromatography procedures using Sephadex G-100 Column (2.5× 100cm) as demonstrated by Manisavagan and Oh (2015).

Effect of temperature and pH on fucoidanase activity

The effect of temperature on the activity of fucoidanase was investigated at temperatures ranging from 30 to 70 ºC using 1% SVF in 0.9 mL 0.2M acetate buffer (pH 5.0) and 0.1 mL purified. Similarly, the effect of pH on the purified enzyme was investigated over a pH range of 3–8 using 0.2M sodium acetate buffer (pH 3.0 to 5.0) and 0.2M phosphate buffer (pH 5.0 to 8.0). Thermal stability of the fucoidanase preparation was further investigated by measuring residual activities of the enzymes at two hour interval during incubation at 55 ºC for 24 h while pH stability of the enzyme was measured by determining the residual activities after incubating the fucoidanase in a pH range of 3.5–7.0 using 0.2M sodium acetate buffer (pH 3.0–5.0) and 0.2 M phosphate buffer (pH 6.0–7.0) for 1 h at 55 ºC.

Effect of different metal ions on fucoidanase Activity

The effects of several metal ions on fucoidanase activity were examined in reaction mixtures supplemented with Na, K, Mg, Cu, Zn, Mn, Ca, and Fe at 0.5 mM concentration on the activity of the fucoidanase from *Aspergillus flavus* FS018, that was investigated at the optimal pH and temperature with *Sargassum vulgare* fucoidan (1.0%) as the substrate, as previously reported by Kim *et al*. (Kim et al., 2015).

Substrate specificity of fucoidanase and kinetics

The substrate specificity of the fucoidanase, produced by *Aspergillus flavus* was investigated using dextran, laminarin, alginate, starch, two commercial fucoidans extracted from *Fucus vesiculosus* (FVF) and *Undaria pinnatida* (UPF) and *Sargassum vulgare* (SVF). An aliquot of 0.9 ml of each of the substrate (1% fucoidan from dissolved with 0.2M acetate buffer) was mixed with 0.1 mL of enzyme extract. The reaction was performed at the optimum, and the mixture was incubated at the optimum temperature and pH for enzyme activity for 1hr. The amount of reducing sugar liberated from the polysaccharide substrate was thereafter measured as described earlier. The enzyme kinetics was investigated using commercial fucoidan (FVF and UPF) and *Saggassum fucoidan* (SVF) at the optimum pH and temperature of enzyme activity with substrate concentration from 0 to 100mM and thereafter the enzyme activity was investigated as stated earlier. The K_m, V_{max} values were evaluated by fitting the experimental data to the Michaelis-Menten model using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego California USA.

Estimation of molecular weight of fucoidanase enzyme

The molecular weight of the purified fucoidanase produced by *Aspergillus flavus* FS018 was determined using SDS-PAGE with a 7.5% (w/v) polyacrylamide gel containing 20% (w/v) SDS alongside protein standards as markers. The protein was stained by Coomassie bright blue G-250.

Results and Discussion

Although fucoidanases have been isolated and purified from different microorganisms, most of the purified and characterized fucoidanases are mainly from marine sources and information on terrestrial microorganisms acting over sulfated-polysaccharide is scarce. Furthermore, the marine fucoidanases reported have low activity and this has limited the industrial application (Gomaa et al., 2018). Hence, the search for microorganisms with high fucoidanase activity remains a challenge. In this study fucoidanase from a recently isolated *Aspergillus flavus* FS018 with high activity (18.7 U/mL) towards the hydrolysis of fucoidan was purified and characterized. To the best of our knowledge this fucoidanase activity is among the highest reported for any microorganism in literature (Rodríguez-Jasso et al., 2008; Silchenko et al., 2013; Kusaykin et al., 2016; Gurpilhares et al., 2016; Wang et al., 2016; Gomaa et al., 2018). The organism was further used in solid state fermentation to ferment dried sterile *Sargassum* powder as substrate after which the fucoidanase was purified using ammonium sulfate procedures and column chromatography. After 5.76 folds of purification, a yield of 74.8% was obtained as shown in Table 1.

The properties of the purified fucoidanase produced by *Apsergillus flavus* FS018 in solid state fermentation was investigated taking the effect of temperature, pH, and metal ions on enzyme activity into consideration. Furthermore, substrate specificity and enzyme kinetics were also determined.

Table 1. Fucoidanase activity of *A. flavus* FS018 at different steps of purification

Purification step	Total Protein (mg/mL)	Total Activity (U/mg)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude	158.4	1358	8.6	-	-
$(NH_4)_2SO_4$ Precipitation	112	1266	11.3	1.31	93.2
Dialysis	44.6	1155	25.8	3.0	85.1
Gel filtration	20..5	1017	49.6	5.76	74.8

Effect of temperature on fucoidanase activity

The effect of temperature on the activity of *Aspergillus flavus* FS018 fucoidanase was measured by incubating the enzyme at the varying temperatures for 10 minutes at pH 4.5 and the results presented in Fig. 1. This result showed that enzyme had maximum activity of 20.8 U/mL at 55 ºC. This optimum activity at 55 ºC is within the optimum temperature range reported for fucoidanases in literature (Kusaykin et al., 2015; Gurpilhares et al., 2016; Wang et al., 2016). This high temperature optimum could be advantageous when considering the use of the enzyme at slightly elevated temperatures (Garuba and Onilude, 2018). The ability of the fucoidanase to resist thermal changes over a period of time was investigated by measuring residual activities of the enzymes at two hour interval during incubation at 55 ºC for 24 h and the results are presented in Fig. 2. The enzyme retained 100% of its activity at the optimum temperature of 55 ºC for 8 hours beyond which the relative activity dropped to 96% when incubated between 8 and 14 hours. The enzyme still retained about 52% of its activity at 24 hours of incubation (Fig. 2). These observations suggest that this fucoidanase can function at higher temperatures. Industrial processes at elevated temperature are considered advantageous as this decreases microbial contamination in large scale industrial reactions of prolonged durations (Ahmed et al., 2009). This enzyme could also find application in the complete saccharification and hydrolysis of sulfated polysaccharides-containing residues which requires a longer reaction time; often associated with the contamination risks over a period of time. Therefore, the hydrolytic enzymes are well sought after being active at higher temperatures as well as retaining stability over a prolonged period of processing at a range of temperatures. The high temperature enzymes also help in enhancing the mass-transfer and reduction of the substrate viscosity (Qianqian et al., 2011).

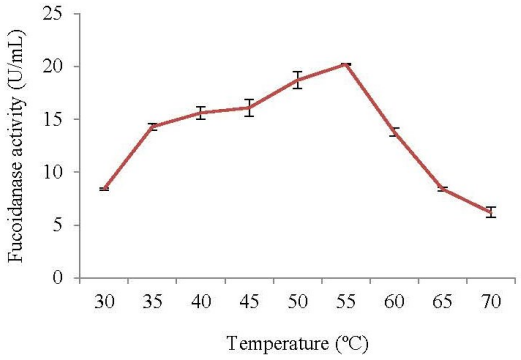

Figure 1. Effect of temperature on the activity of fucoidanase produced by *Aspergillus flavus* FS018.

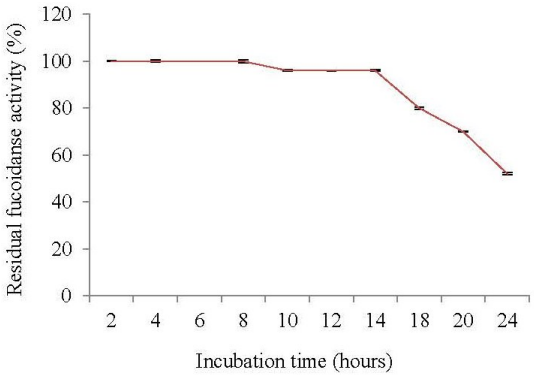

Figure 2. Thermal stability of fucoidanase produced by *Aspergillus flavus* FS018.

Effect of pH on fucoidanase activity and pH stability

The effect of pH on the activity of fucoidanase was also investigated by incubating the enzyme at varying pH at 50 ºC for 10 minutes and the results are presented in Fig. 3. The results showed that the enzyme had the highest activity of 17.2 U/mL at pH 5.0. Below and above this, a decrease in fucoidanase activity was observed (Fig. 3). The effect of different pHs on the stability as shown in Fig. 4 revealed that the fucoidanase enzyme retained over 70% of its activity over a broad range of pH (4-7). The optimum pH reported in this study is similar to those of other fungi isolated from marine sources (Berka et al., 2011; Wu et al., 2011). Loss of activity beyond the optimum pH could be due to changes in the structural configuration of the protein molecule resulting from the change in ionic properties of the substrate solution. These results suggest that the fucoidanase is active and stable over a wide range of pHs which is typical of fucoidanases especially from marine bacteria and invertebrate sources (Silchenko et al., 2013).

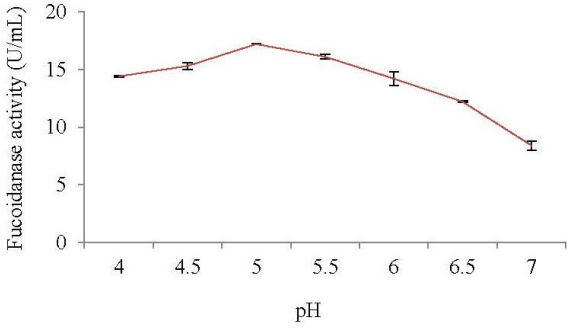

Figure 3. Effect of pH on activity of fucoidanase produced by *Aspergillus flavus* FS018.

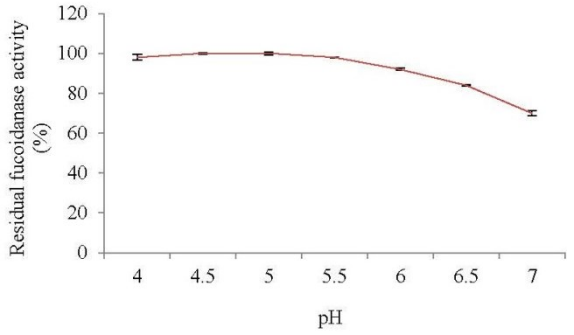

Figure 4. pH stability of fucoidanase produced by *Aspergillus flavus* FS018.

Effect of metal ions on fucoidanase activity

The importance of metal ions on the activity of the fucoidanase was also investigated and the results are presented in Fig. 5. The results demonstrated that magnesium and calcium ions had a stimulatory effect on the fucoidanase with a relative activity of 112.7 U/mL and 118.6 U/mL, respectively while the other ions (Na^+, K^+, Fe^{2+}, and CU^{2+}), investigated in this study showed some inhibitory effect on the activity of this Fucoidanase. This result suggests that the Fucoidanase from this *Aspergillus flavus* FS018 does not require Na^+ and K^+ for the hydrolysis of fucoidan as the case for several fucoidanase was isolated from different marine organisms (Kim et al., 2015). On the other hand, this fucoidanase having some stimulatory activity in the presence of magnesium and calcium ions could suggest that the enzyme require some divalent metal ions for the hydrolysis of *Sarggasum* fucoidan.

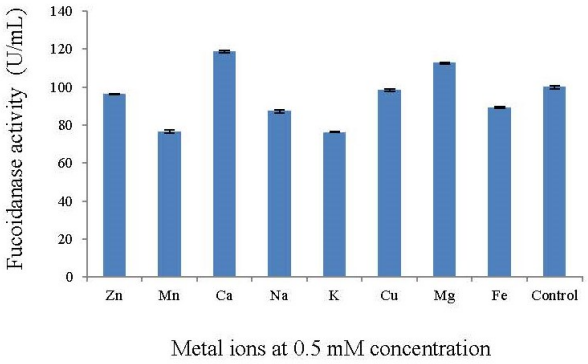

Figure 5. Effect of different metal ions on the activity of fucoidanase produced by *Aspergillus flavus* FS018.

Substrate specificity of fucoidanase FS018

Substrate specificity of the *Aspergillus flavus* FS018 fucoidanase was investigated using different substrates as this can help determine the enzyme structure and physiological functions as well as addressing the key residues involved in substrate binding and catalysis. The results presented in table 2 shows that fucoidanase from *Aspergillus flavus* FS018 had strong affinity for commercial fucoidan1 (from *Fucus vesiculosus*) with an activity of 148±0.5% (compared to control), an activity of 139±0.6% (compared to control) for commercial fucoidan from *Undaria pinnatida* (commercial fucoidan 2) and an activity of 120±0.8% (compared to control) for alginate while the other substrates investigated decreased the activity of this fucoidanase (Table 2). These results could suggest that the enzyme is active for fucoidan consisting of α-1→4 and α-1→3 glycoside bonds alternating in the main chains and galactofucan group (Khanh et al., 2019).

Table 2. Substrate specificity of fucoidanase produced by terrestrial *Aspergillus flavus* FS018 isolated from agricultural soil

Substrate	Relative activity (%)
Dextran	32±0.5*
Alginate	120±0.8
Starch	34±1.2
Laminarin	68±1.8
Commercial fucoidan1	148±0.5
Commercial fucoidan2	139±0.6
**Control	100±1.4

Data are means of three replicates ± Standard Error of Mean
**Control-Fucoidan from *Sargassum vulgare*

Estimation of kinetic parameters

The kinetic parameters of the fucoidanase from *Aspergillus flavus* using commercial fucoidan1, commercial fucoidan2 and *Sargassum* fucoidan showed that the K_m = 1.4, 1.6, and 1.9 mM, while V_{max} = 0.34, 0.36, and 0.38 mg/min, respectively (Data not shown). From these data, Kcat of the enzyme against commercial fucoidan1, commercial fucoidan2, and *Sargassum* fucoidan were found to be 0.24 S^{-1}, 0.22S^{-1}, and 0.20 S^{-1} (V_{max}/K_m), respectively (Table 3). These data suggest that the enzyme has high affinity for these substrates thereby becoming quickly saturated with substrate, and acting at a more or less constant rate, regardless of variations in the concentration of substrate within the physiological range.

Table 3. Kinetic parameters of fucoidanase from *Aspergillus flavus* FS018 with fucoidans isolated from various sources

Substrate	Kcat (S^{-1})	K_m (mM)	V_{max} (mg/min)	Kcat/Km(S^{-1} mM^{-1})
CF1	0.24	1.4	0.34	0.17
CF2	0.22	1.6	0.36	0.14
SVF	0.20	1.9	0.38	0.11

CF1- Commercial fucoidan1
CF2- Commercial fucoidan2
SVF- *Sargassum vulgare*

Molecular weight determination

SDS-PAGE analysis of the purified enzyme (purified by gel chromatography procedures using Sephadex G-100 Column (2.5× 100cm)) as presented in Fig. 6 showed that the enzyme is a monomeric enzyme molecule with an estimated molecular weight of 70 kDa. Although there is a dearth of information on the molecular weight of fucoidanase from terrestrial microorganism, the existing literature shows there exists a great variation in the molecular weight of fucoidanase produced by various organisms. The molecular weight of this fucoidanase from terrestrial *Aspergillus flavus* FS018 in this study is similar to those of the other marine fungi (Qianqian et al., 2011; Wu et al., 2011) and in the range of molecular weight of fucoidanase purified from different marine organisms (39-200 kDa) as reported earlier (Wu et al., 2011). The variation observed in molecular weight of fucoidanase from different microorganisms could be as a result of the variation in the differential glycosaylation of the fucoidanase protein.

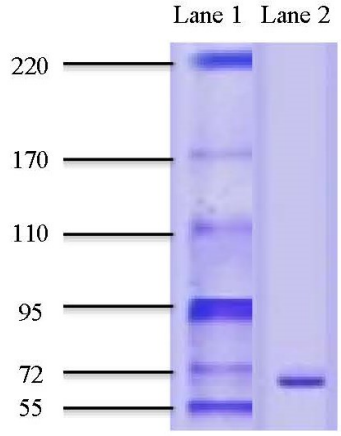

Figure 6. Electrophoretogram of the SDS-PAGE analysis of the purified (purified by gel chromatography using Sephadex G-100 Column) fucoidanase produced by *Aspergillus flavus* FS018; Lane 1 - Molecular weight makers (kDa), Lane 2- purified fucoidanase.

Conclusion

Conclusively, this paper presents the properties of purified thermostable fucoidanase from terrestrial *Aspergillus flavus* FS018. To the best of our knowledge, this is one of the first studies reporting the properties of fucoidanase from terrestrial microorganism. The high optimum temperature of 55 ºC, optimum pH of 5 and estimated K_m and V_{max} of 1.9 mM and 0.38 mg/min respectively with fucoidan from *Sargassum vulgare* makes this enzyme an ideal candidate for the potential hydrolysis of fucoidan consisting of α-1→4 and α-1→3 glycoside bonds in the main chains and also galactofucans group. However, efforts are been made to optimize the production by the *Aspergillus flavus* FS018 and also to characterize the products of fucoidan hydrolysis by this enzyme.

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Competing Interests

The authors declare that they have no conflict of interest.

Authors' Contribution

This work was carried out in collaboration with all the authors. E. O. Garuba designed the study, E. O. Garuba and A.P. Adedeji wrote the protocol and performed the work, A. P. Adedeji and E.O. Garuba wrote the first draft of the manuscript. A. A. Onilude supervised the study. All the authors read and approved the final manuscript.

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