ESTIMATION OF CITRIC ACID PRODUCTION BY FUNGAL FERMENTATION OF SOME SPOILED FRUITS

[1]Malik, A. I., [2]Osesua, B. A. and [1]Fasiku, O. O.

[1]Biology Unit, School of Preliminary and Remedial Studies, Waziri Umaru Federal Polytechnic, P.M.B. 1034, Birnin-Kebbi, Kebbi State, Nigeria.
[2]Chemistry Unit School of Preliminary and Remedial Studies, Waziri Umaru Fed. Poly. Birnin-Kebbi, Kebbi State, Nigeria.
Email: osesuaa@yahoo.com

ABSTRACT

Spoiled banana, orange and pineapples were fermented using *Aspergillus niger* and *Aspergillus flavus* for citric acid production. The production efficiency of these spoiled fruits was determined by comparing them with the healthy fruits of the same kind (control). The ability of the fermenting fungi was determined by screening the fungal isolates for citric acid production using Czapekdox agar and bromocresol green as indicator. Estimation of citric acid produced was done by pyridine-acetic anhydride method. Of the three spoiled fruits used, spoiled pineapple gave the highest citric acid yield of 6.8g/l (1.4%) and 2.2g/l (0.5%) with both *Aspergillus niger* and *Aspergillus flavus* respectively at t-value of 2.50 and p=0.05. Healthy orange gave the highest citric acid yield of 59.1 (21.9%) and 3.7 (0.4%) with both *Aspergillus niger* and *Aspergillus flavus* respectively at t-value of 3.59 and p=0.05. Citric acid yield from healthy fruits (control) was higher than the citric acid yield from spoiled fruits.

Keywords: Spoiled fruits, healthy fruits, citric acid, fermenting fungi and fungal isolates**.**

INTRODUCTION

Citric acid (2-hydroxy-1,2,3-propane tri-carboxylic acid) is a versatile chemical having a wide range of applications in food industries, pharmaceutical industries and cosmetic industries (Khadijah and Mazharuddin, 2011). Citric acid is used in the food and beverage industry to flavour fruit juices, candy ice cream and marmalade. In the pharmaceutical industry, citric acid is used as a preservative for stored blood, tablets, ointments and cosmetic preparations (Penniston *et al*., 2008). The cleansing characteristic of citric acid makes it ideal for use as industrial cleaner (Dhillon *et al*., 2011).

There is a great worldwide demand for citric acid consumption due to its low toxicity when compared with other acidulants used mainly in the pharmaceutical and food industries (Radwan *et al*., 2010). Approximately, 75% commercial use of citric acid is for food and 12% for pharmaceutical industries (Haq *et al*., 2002). Citric acid is an important organic acid produced by microbial fermentation process. It exists as an intermediate in the citric acid cycle, when carbohydrates are oxidized to carbon dioxide (Haq *et al*., 2002). Commercial production of citric acid is generally by submerged fermentation of sucrose or molasses using the filamentous fungus, *Aspergillus niger,* or synthetically from acetone or glycerol (Haq *et al*., 2004).

The worldwide demand of citric acid is about 6.0×10^5 tons per year and it is bound to increase day by day (Karaffa and Kubicek, 2003). With an estimated annual production of 1,000,000 tons, citric acid is one of fermentation products with the highest production level worldwide. The food industry consumes about 70% of total citric acid produced, while other industries consume the remaining 30% (Kapoor *et al*., 2004). From this point of view, it is necessary to use inexpensive and readily-available raw materials for industrial production processes. At present, a variety of agro-industrial residues and by-products are used as substrates for citric acid production such as cassava bagasse (Kumar and Jain, 2008) coffee husk, wheat bran, apple pomace (Vandenberghe *et al*., 2004), pineapple waste (Imandi *et al.,* 2008).

Fungi are very effective and efficient biodegraders because of the wide range of extra-cellular enzymes they produce, which are capable of degrading complex polymers (Magnuson and Linda, 2007).

Fruits and vegetables are more prone to spoilage than cereals due to their nature and composition, and this spoilage occurs at the time of harvesting, handling, transportation, storage, marketing and processing, resulting in waste (Harender and Guleria, 2007).Due to inadequate storage and processing facilities of fruits during their harvest season, large percentage of these fruits may be left unconsumed and as a result, becomes deteriorated by spoilage microorganisms. This could contribute to environmental pollution and also result in economic losses. Likewise, many fruit wastes are dumped indiscriminately after extracting the edible portion and this uncontrolled activity leads to environmental pollution with the resultant health hazard to the populace.

MATERIALS AND METHODS

Samples Collection and Processing

Fruits (i.e., healthy and spoiled orange, banana and pineapple) were purchased from Fruit Market in Sokoto. Some of the spoiled fruits selected were either physically damaged with wounds or infected by spoilage microorganisms, by the physical presence of appresorium on their skin and localized soft rots while healthy fruits without any blemish damage or being excessively ripened were carefully selected. Spoiled and healthy fruits were transported in separate sterile polythene bags to the Microbiology laboratory of Usmanu Danfodiyo University.

The unpeeled skin of each fruit was sterilized with 90% ethanol (Akinmusire, 2011). Each fruit was prepared by peeling the entire skin and cutting the pulp into mesh sizes of approximately 2mm with the aid of a scarpel and 5g was weighed using Mettler PE 200 weighing balance and used as substrate in each Erlenmeyer flask employed for the fermentation process. A similar procedure was carried out on the healthy fruits, which served as control. This procedure was carried out according to Khadijah and Mazharrudin (2011).

Isolation and Identification of Fungi from Soil

Aspergillus niger and *Aspergillus flavus* were collected from the Mycology Laboratory of Usmanu Danfodiyo University, Sokoto. They were revived by subculturing them on fresh media in order to check their viability. Screening of these organisms for citric acid production was carried out according to the method of Sikander (2005). *Aspergillus niger* and *Aspergillus flavus* showed yellow colour zones of 2.1mm and 0.9mm respectively around their mycelia

Fermentation Medium Preparation

The fermentation medium was formulated according to the method of Kuforiji *et al*. (2010). This was done by weighing 100ml distilled water, 0.5g/lNH_4NO_3, 0.2g/l $KH_2PO_4$and 5g of each fruit into 250ml Erlenmeyer flask. Flasks were cotton plugged and sterilized at 121^0C for 15minutes.

Inoculum Preparation

Spores of *Aspergillus niger* and *Aspergillus flavus* were cultivated and standardized according to the method of Jin-woo (2004). Each fungal isolate (2mm) was inoculated on to the surface of already prepared PDA slants and incubated for five days at 30^0C. Spore inoculum was prepared by adding 3ml of sterile distilled water to each slant bottle containing isolates. The bottle was gently agitated and 1m of the suspension was used to inoculate each of the fermenting flasks, which were incubated at 30^0C for fermentation.

Estimation of Citric Acid Yield

The procedure for citric acid estimation by pyridine-acetic anhydride method was carried out according to Khadijah and Mazharrudin (2011). Anhydrous citric acid was used as standard for sample's citric acid estimation. Pyridine of 1.3ml and 5.7ml acetic anhydride were used. Absorbance was determined using UV spectrophotometer. Statistical analysis of result was carried out using Student's t-test

RESULTS

Aspergillus flavus and *Aspergillus niger* were screened for citric acid producing-ability and employed to produce citric acid from both healthy and spoiled orange, pineapple and banana.

Figure 1 shows the initial and final pH values of the broth containing each substrate with *Aspergillus flavus* and *Aspergillus niger*. The initial pH values for healthy banana, orange and pineapple were 5.26, 4.45 and 4.22 respectively. Final pH values for healthy banana, orange and pineapple fermented with *Aspergillus niger* were 2.96, 2.31 and 2.85 respectively while 3.51, 3.25 and 3.30 were recorded respectively for banana, orange and pineapple fermented with *Aspergillus flavus*. On the other hand, initial pH values for spoiled banana, orange and pineapple were 4.51, 4.17 and 3.84 respectively. Final pH values for the spoiled banana, orange and pineapple with *Aspergillus niger* were 4.42, 4.07 and 3.72 for banana, orange and pineapple respectively while 4.02, 3.62 and 3.35 were recorded for banana, orange and pineapple with *Aspergillus flavus*.

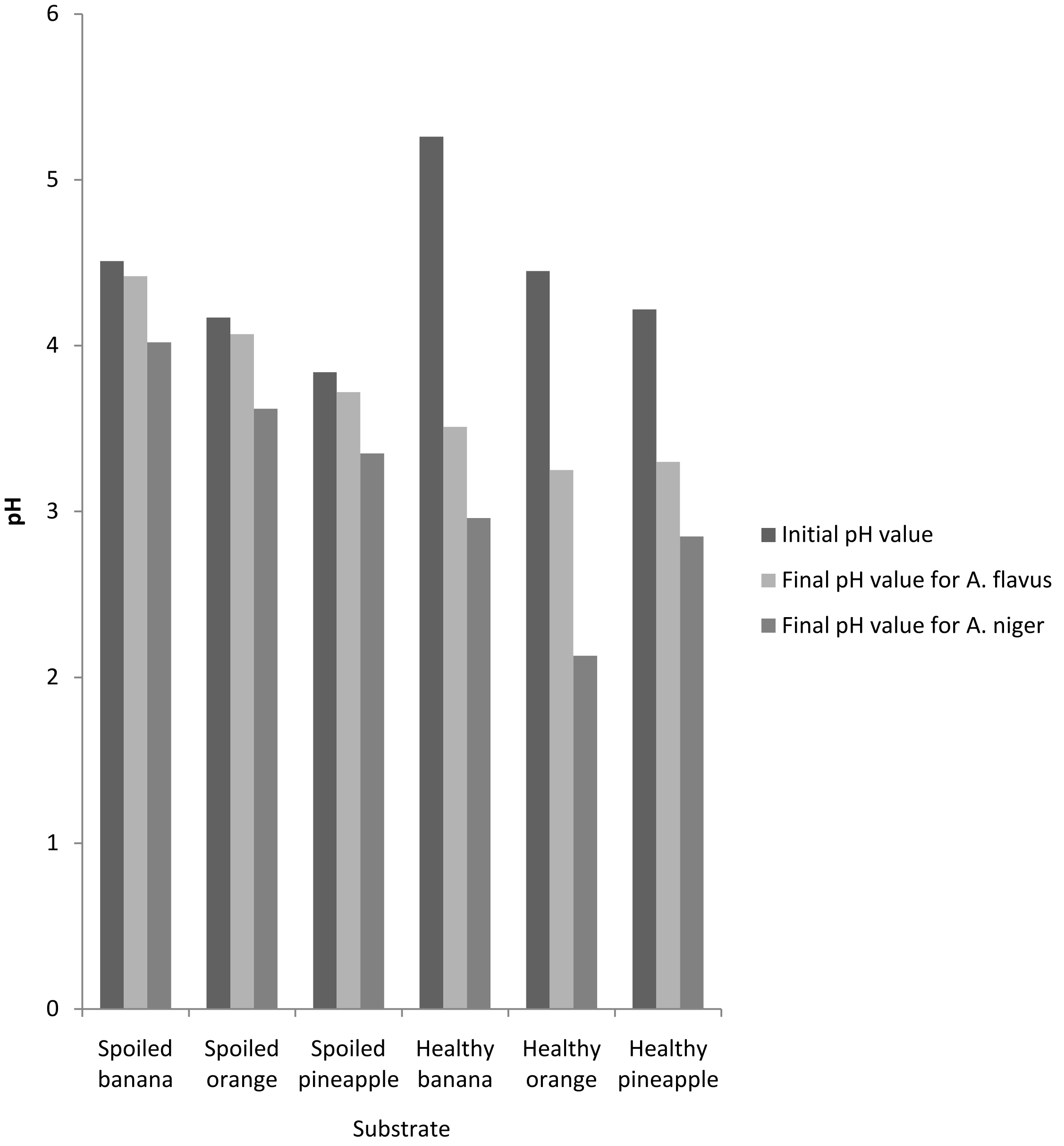

Figure 1: Initial and final pH of broth containing each substrate with *Aspergillus flavus* and *Aspergillus niger*

The results showed that the initial pH values of broth containing spoiled fruits were lower than the initial pH values of broth containing healthy fruits at the start of the fermentation process. At the end of the fermentation (144hours), pH values of broth containing healthy fruits were lower than pH values of broth containing spoiled fruits for both *Aspergillus flavus* and *Aspergillus niger*.

Table 1 shows the comparison of citric acid yield by *Aspergillus niger* and *Aspergillus flavus* from spoiled fruits. The citric acid-producing ability of *Aspergillus niger* and *Aspergillus flavus* from spoiled orange, spoiled pineapple and spoiled banana was compared and the result was presented. For the spoiled orange, *Aspergillus niger* produced 3.7g/l (0.4%) citric acid while *Aspergillus flavus* produced 0.7g/l (0.1%) citric acid. This result at t-value of 1.60

shows there is no statistical significant difference at 0.05 in citric acid yield between *Aspergillus niger* and *Aspergillus flavus,* i.e., there is low citric acid yield by *Aspergillus niger* and *Aspergillus flavus* from spoiled orange.

For the spoiled pineapple, *Aspergillus niger* produced 6.8g/l (1.4%) citric acid while *Aspergillus flavus* produced 2.2g/l (0.5%) citric acid. This result at t-value of 2.50 shows there is no statistical significant difference at 0.05 in citric acid yield between *Aspergillus niger* and *Aspergillusflavus,* i.e., there is low citric acid yield by *Aspergillus niger* and *Aspergillus flavus* from spoiled pineapple.

For spoiled banana, *Aspergillus niger* produced 3.0g/l (0.1%) citric acid while *Aspergillus flavus* produced 1.5g/l (0.05%) citric acid. This result at t-value of 0.73 shows there is no statistical significant difference at 0.05 in citric acid yield between *Aspergillus niger* and *Aspergillus flavus,* i.e., there is low citric acid yield by *Aspergillus niger* and *Aspergillus flavus* from spoiled banana.

Table 1 Comparison of citric acid yield by *Aspergillus niger* and *Aspergillus flavus* from spoiled fruits

Duration (Hours)	Citric acid yield from spoiled fruits by the fungi (g/l)					
	Spoiled orange		Spoiled pineapple		Spoiled banana	
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus flavus*
48	2.3	0.5	2.1	0.8	0.9	0
96	1.2	0.2	3.4	0.8	2.0	1.3
144	0.2	0	1.3	0.6	0.1	0.2
Total citric acid yield	3.7 (0.4%)	0.7 (0.1%)	6.8 (1.4%)	2.2 (0.5%)	3.0 (0.1%)	1.5 (0.05%)

p-value=0.05 df= 4, t= 1.60 t= 2.50 t= 0.73

Table 2 shows the comparison of citric acid yield between healthy and spoiled fruits by *Aspergillus niger*. Citric acid produced from healthy orange is 59.1g/l (21.9%) while 3.7g/l (0.4%) citric acid was produced from spoiled orange. This result at t-value of 3.59 shows there is statistical significant difference at 0.05 in citric acid produced from healthy and spoiled orange, i.e., healthy orange produced more citric acid than spoiled orange by *Aspergillus niger*.

Citric acid produced from healthy pineapple is 51.3g/l (14.8%) while 6.8g/l (1.4%) citric acid was produced from spoiled orange. This result at t-value of 3.51 shows there is statistical significant difference at 0.05 in citric acid produced from healthy and spoiled pineapple, i.e., healthy pineapple produced more citric acid than spoiled pineapple by *Aspergillus niger*.

Citric acid produced from healthy banana is 33.9g/l (6.1%) while 3.0g/l (0.1%) citric acid was produced from spoiled banana. This result at t-value of 3.59 shows there is statistical significant difference at 0.05 in citric acid produced from healthy and spoiled banana, i.e., healthy banana produced more citric acid than spoiled banana by *Aspergillus niger*.

Table 3 shows the comparison of citric acid yield between healthy and spoiled fruits by *Aspergillus flavus*. Citric acid produced from healthy orange is 4.8g/l (1.8%) while 0.7g/l (0.1%) citric acid was produced from spoiled orange. This result at t-value of 1.01 shows there is no statistical significant difference at 0.05 in citric acid produced from healthy and spoiled orange, i.e., citric acid yield by *Aspergillus flavus* from healthy and spoiled orange was low.

Citric acid produced from healthy pineapple is 3.0g/l (0.9%) while 2.2g/l (0.5%) citric acid was produced from spoiled pineapple. This result at t-value of 0.42 shows there is no statistical significant difference at 0.05 in citric acid produced from healthy and spoiled pineapple, i.e., citric acid yield by *Aspergillus flavus* from healthy and spoiled pineapple was low.

Citric acid produced from healthy banana is 2.9g/l (0.5%) while 1.5g/l (0.05%) citric acid was produced from spoiled banana. This result at t-value of 0.63 shows there is no statistical significant difference at 0.05 in citric acid produced from healthy and spoiled banana, i.e., citric acid yield by *Aspergillus flavus* from healthy and spoiled banana was low.

Table 2 Comparison of citric acid yield between healthy and spoiled fruits by *Aspergillus niger*

Duration (Hours)	Citric acid yield (g/l) between healthy and spoiled fruits by *Aspergillus niger*					
	Orange		Pineapple		Banana	
	Healthy	Spoiled	Healthy	Spoiled	Healthy	Spoiled
48	19.2	2.3	15.5	2.1	12.3	0.9
96	28.8	1.2	24.8	3.4	15.1	2.0
144	11.1	0.2	11.0	1.3	6.5	0.1
Total citric acid yield	59.1 (21.9%)	3.7 (0.4%)	51.3 (14.8%)	6.8 (1.4%)	33.9 (6.1%)	3.0 (0.1%)

p-value=0.05 df= 4, t= 3.59 t= 3.51 t= 3.97

Table 3 Comparison of citric acid yield between healthy and spoiled fruits by *Aspergillus flavus*

Duration (Hours)	Citric acid yield (g/l) between healthy and spoiled fruits by *Aspergillus flavus*					
	Orange		Pineapple		Banana	
	Healthy	Spoiled	Healthy	Spoiled	Healthy	Spoiled
48	0.2	0.5	0	0.8	0.5	0
96	4.3	0.2	1.1	0.8	2.2	1.3
144	0.3	0	1.9	0.6	0.2	0.2
Total citric acid yield	4.8 (1.8%)	0.7 (0.1%)	3.0 (0.9%)	2.2 (0.5%)	2.9 (0.5%)	1.5 (0.05%)

p-value=0.05 df= 4, t= 1.01 t= 0.42 t= 0.63

DISCUSSION

In Figure 1, pH was determined from all the incubated samples and there was progressive decrease in pH values as incubation time increased. Initial broth pH values obtained for spoiled banana, orange and pineapple decreased from 4.51, 4.17 and 3.84 to 4.42, 4.07 and 3.72 for *Aspergillus niger* and 4.02, 3.62 and 3.35 for *Aspergillus flavus* respectively. Initial broth pH values obtained for healthy banana, orange and pineapple decreased from 5.26, 4.45 and 4.22 to 2.96, 2.31 and 2.85 for *Aspergillus niger* and 3.51, 3.25 and 3.30 for *Aspergillus flavus* respectively. This implies that during fermentation, broth pH value decreases as fermentation time increases until the microorganism stops producing citric acid. This is in agreement with the findings of Kareem *et al.*, (2010) who reported that citric acid production occurs after 24 hours of fermentation and as incubation time increased, more citric acid was produced while the pH values decreased. Thus, the drop in pH observed during the fermentation process was due to the formation and accumulation of citric acid. Lower initial broth pH was also observed for spoiled banana, orange and pineapple, which were 4.51, 4.17 and 3.84 respectively, when compared to that of healthy banana, orange and pineapple, which were obtained as 5.26, 4.45 and 4.22 respectively. This could be due to the production of some organic acidic compounds as a result of microbial spoilage. Final broth pH values for healthy banana, orange and pineapple, which were obtained as 2.96, 2.31 and 2.85 respectively for *Aspergillus niger* and 3.51, 3.25 and 3.30 for *Aspergillus flavus,* after 144 hours of fermentation were more reduced than the final broth pH values for spoiled banana, orange and pineapple, which were obtained as 4.42, 4.07 and 3.72 respectively for *Aspergillus niger* and 4.02, 3.62 and 3.35 for *Aspergillus flavus*. This could be due to higher concentration of fermentable substrate in healthy fruits. This supports the report of Chuku *et al*. (2008) that there is a variation in the susceptibility of fruits, which is due largely to the differential chemical composition such as pH and moisture contents.

In table 1, there was a higher yield of citric acid by *Aspergillus niger* from spoiled orange, pineapple and banana (3.7g/l, 6.8g/l and 3.0g/l respectively)when compared with the yield of citric acid by *Aspergillus flavus* from spoiled orange, pineapple and banana (0.7g/l, 2.2g/l and 1.5g/l). High yield of citric acid by *Aspergillus niger* could be explained in terms of its production consistency. Consistency in the production of citric acid by *Aspergillus niger* was observed all through the fermentation processes unlike in *Aspergillus flavus* where there was no citric acid yield at the initial period of fermentation for the spoiled banana. This implies that the *Aspergillus niger* strain better adapts to the medium, which enhances its early citric acid production. This is in line with the findings of Khadijah and Mazharuddin (2011) that *Aspergillus niger* hydrolyzes cellulose present in substrates to simple sugars by saccharification process to produce citric acid by fermentation. This is also in line with the findings of Alagarsamy and Nullusamy (2010) that the benefits of using *Aspergillus niger* are its ease of handling, its ability to ferment a wide range of inexpensive raw materials and high yield. This observation is also in line with Carlos *et al.* (2006) who reported that *Aspergillus niger* has remained the organism of choice for commercial production of citric acid because it produces more citric acid per unit time. Thus, *Aspergillus niger* remains the superior industrial species for the production of citric acid. This was reported by Kirimura*et al*. (2000). It also goes in line with the findings of Makut and Ade-Ibijola (2012), where *Aspergillus niger* produced the highest quantity of citric acid from different soil fungal isolates.

In Table 2, citric acid yield from spoiled orange, pineapple and banana, which were obtained as 3.7g/l, 6.8g/l and 3.0g/l respectively, was lower than citric acid yield from healthy orange, pineapple and banana, which were obtained as 59.1g/l, 51.3g/l and 33.9g/l respectively by *Aspergillus niger*. This could be due to the low concentration of fermentable substrates as a result of its conversion to alcohol and other organic compounds by spoilage microorganisms before the fruits were fermented for citric acid production. This implies that increase in fermentable substrate concentration enhances high yield of citric acid by fermentation. This is in line with the work of Khadijah and Mazharuddin (2011) who reported increase in the citric acid yield with *Aspergillus niger* grown in flasks containing orange peels as substrate with high sugar concentration in the media, than *Aspergillus niger* grown in flasks containing orange peels as substrate with low sugar concentration in the media. In addition, it was observed that spoiled pineapple yielded 6.8g/l of citric acid while spoiled orange and spoiled banana yielded 3.7g/l and 3.0g/l respectively. This could be due to difference in degree of spoilage of the fruits, i.e. spoilage could have resulted in the reduction of the amount of fermentable substrates in orange and banana more than pineapple.

In Table 3, low or non-production of citric acid up to 48hours of fermentation was observed in healthy pineapple and spoiled banana fermented by *Aspergillus flavus*. This may be due to biomass production at the start of the fermentation process and not necessarily for citric acid production. This was characterized by the adhesion of the microorganisms to each of the small-sized substrates in the fermenting flask with a subsequent build up of mycelia around the substrates. This implies that at the initial period of citric acid fermentation, lag phase was longer for *Aspergillus flavus* spores than *Aspergillus niger s*pores. *Aspergillus niger* better adapts to the medium with early citric acid production. This observation is in line with Khadijah and Mazharuddin (2011) who reported that spore suspension inoculum utilized substrate in the fruits for two purposes, i.e., for growth (into mycelium) and for fermentation of citric acid.

Conclusion

This research shows that spoiled pineapple produced the highest citric acid of the three spoiled fruits used and *Aspergillus niger* better ferment the substrates used, giving a higher citric acid yields than *Aspergillus flavus*.

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