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RESEARCH ARTICLE

Effect of pre-culture incubation duration on the physicochemical and microbial properties of vegan yogurt

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Abstract

At present, the consumption trend of plant-based nutritional products is becoming increasingly widespread, particularly among individuals with lactose intolerance or milk allergies. This study aimed to develop a plant-based yogurt using purple rice supplemented with soymilk, focusing on optimizing fermentation efficiency and product quality. The effects of varying commercial yogurt starter culture concentrations (0.5 %, 1.0 %, 1.5 % and 2.0 %) and pre-culture incubation durations (8, 16, 24, 32, 40 and 48 hr) were investigated. Bacterial density, fermentation time, lactic acid production and water-holding capacity of the yogurt were checked as the observed criteria. With a 1.5 % inoculum ratio pre-culture incubated within 32 hr, which was selected as the optimal condition ensuring between fermented efficiency and yogurt quality. Applying this condition, the bacterial concentration achieved 5.81×10^8 (cfu/mL), the fermentation duration was shortened to 360 min and the water-holding capacity (WHC) reached its maximum value at 94.66 %, presenting a well-formed gel texture with high flocculation and enhanced sensory attributes. This paper provided a practical approach for economical production of high-quality plant-based yogurt from unutilized crops.

Keywords: fermentation efficiency; incubation duration; inoculum ratio; plant-based yogurt; water holding capacity

Introduction

The global plant-based dairy business is growing rapidly because more people are learning about health, the environment and moral issues. The number of plant-based dairy substitutes has been increasing for more than ten years and is still going up because of lactose intolerance, milk allergies and the demand for lower-calorie, nutrient-rich solutions (1). It is predicted that this will increase from 7.4 % to more than 18.5 % of the total market for milk-based items. Soybeans, almonds, coconuts, rice and oats can all be used to make vegan dairy products. The most popular goods made from soybeans are soymilk extract and analogues of cheese and yogurt (2). Soybean is an important grain in human nutrition since it has a lot of nutrients and may help stop free radicals, lower the risk of obesity, heart disease, chronic inflammation, diabetes and cancer (3).

Another common element in vegan foods is purple rice. Purple rice is rich in fiber, iron, calcium, vitamins (B_1, B_2, E, C and D) and minerals (4). Anthocyanin is abundant in purple rice, the bran extract is cyanidin 3-glucoside, which has a hypoglycemic effect through regulating lipogenic enzyme activity (5). In addition, anthocyanin-rich extract from purple rice has been shown to prevent hyperlipidemia and diabetic syndrome in fructose-fed rats (6).

Yogurt has long been considered as a functional food since it provides a unique balance of nutrients and health benefits. But more people are becoming lactose intolerant, allergic to dairy or following a plant-based diet, which has made plant-based yogurt a healthier and more popular option to normal dairy (7). The figures suggest that the global market for plant-based yogurt was worth more than $1.5 billion in 2019 and will be worth around $3 billion by 2026 (8). Plant-based yogurt is manufactured from aqueous extracts of legumes, grains and oilseeds. This gives it different nutritional and chemical qualities than ordinary yogurt made from cow's milk (9-11). Traditional and plant-based yogurts both have several health benefits, such as making digestion easier and reducing bloating. This is because bacterial α-galactosidase breaks down oligosaccharides during fermentation (12, 13). Plant-based yogurts, on the other hand, may offer additional benefits. They are naturally free of cholesterol and bioactive compounds like arabinose, tocopherol and sterols, which are beneficial for health since they fight diabetes, inflammation and free radicals (14, 15).

Fermenting soybean-based foods with lactic acid bacteria is a good method to make them healthier and more useful (16). Probiotic foods, which have lived beneficial microorganisms in them, can aid the host in various ways, such as by making their gut health and immune system healthier (17). Fermentation not only makes food simpler to digest and taste better, but it also generates new bioactive molecules that combat free radicals, obesity and inflammation (18). Fermenting new plant-based components is anticipated to become a major focus in developing the next generation of functional meals (19). This is because more consumers demand plant-based diets.

Soybeans are suitable substrate for lactic acid bacteria to develop because they are abundant in protein (20). But there aren't enough studies on soy-based yogurts to address challenges related to taste, texture, microbial balance and nutrient composition. This is an initial study focused on creating a method for making vegan yogurt using purple rice and soy milk. The major goals are to standardize the amount of commercial starter cultures and discover the ideal time to incubate the pre-culture. This will enhance the fermentation process, speed it up and reduce production costs, all while retaining the yogurt's quality.

Materials and Methods

Materials

Purple rice was of the purple ST variety (Soc Trang, Vietnam), which was purchased from Shrimp Rice Co., Ltd (Vietnam); soybeans were supplied by Tam Nong Co., Ltd (Vietnam).

Yoghurt was made using commercial starter culture consisting of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains, which were obtained from Happy Energy Co., Ltd (Vietnam).

Other analytical-grade chemicals were sourced from Sinopharm Chemical Reagent Co., Ltd (China) and provided by Cemaco Vietnam Co., Ltd., Can Tho, Vietnam with analytical grade.

Sample procedure

A total of 50 mL of *Lactobacillus* MRS broth was sterilized at 121 °C for 20 min and then cooled to 37 °C.

Soymilk preparation

Soybeans were rinsed to remove impurities and soaked for 8 hr to remove the outer layer of soybeans. After that, soybeans were blended using a Blue Stone BLB-6035 blender. The ratio of soybeans to water was 125 g to 600 mL (21). After blending, the mixture was filtered and the filtrate was collected.

Fermentation substrate was made of purple rice; 100 g of purple rice were sorted and rinsed thoroughly. After draining, the rice was added with water at a ratio of 1 part rice to 4 parts water (w/v) and cooked in a cooking pot (TC-NK6D, China) for 40 min. The cooked rice mixture was then blended into a puree and filtered through a 0.5 mm sieve to collect the filtrate.

A 1:1 (v/v) mixture of soy milk and purple rice extract was pasteurized at 85 °C for 15 min. Its total soluble solids content was modified to 14 °Brix using sucrose and cooled to 43 °C for the fermentation.

Experimental design

The experiment was arranged in a completely randomized design with two factors (A) commercial starter culture ratio (0.5, 1.0, 1.5 and 2.0 % w/v) and (B) pre-incubation time (8, 16, 24, 32, 40 and 48 hr). The experiment was carried out with three replicates to collect the starter culture medium and the bacterial density of the starter culture medium was determined.

Experimental methods

Commercial yogurt cultures were added in % (w/v) ratio to MRS culture medium (sampling procedure) and incubated to increase the microbial density at different time intervals (hr). After different incubation periods at 37 °C, colony counts were performed and bacterial density was calculated. Simultaneously, the study performed yogurt fermentation with soymilk mixture and purple rice extract (sampling procedure). 450 mL of purple rice/soybean mixture was supplemented with 50 mL of starter culture medium. The mixture was fermented at 43 °C until the pH reached 4.6, the fermentation time for each yogurt sample was recorded, other characteristics of the yogurt samples including the amount of lactic acid produced and water holding capacity were evaluated.

Microbiological determination

The total lactic acid bacteria (LAB) count (TLC) of yoghurt samples was counted on MRS agar. Diluted samples were spread on plates and incubated at 37 °C for 48 hr (22). The number of LAB was enumerated through the following formula 1:

$$\text{LAB} = \text{num of colonies} \times \frac{1}{\text{dilution factor}} \qquad \text{(Eqn. 1)}$$

Lactic acid determination

Lactic acid was identified through its reaction with iron (III) chloride, forming a yellowish-green iron (III) lactate complex in solution, as described earlier (23). In this method, 50 µL of the lactic acid-containing sample was mixed with 2 mL of a 0.2 % iron (III) chloride solution and stirred. The reaction took place at 25 °C and the color was allowed to stabilize for 15 min before measuring the absorbance at 390 nm. The concentration of lactic acid was determined using a standard calibration curve, $y = 31.1360x + 0.2709$ ($R^2 = 0.9999$), where y denotes the absorbance and x denotes the concentration of solution in the tube.

Water holding capacity determination

Water holding capacity (WHC) was measured by centrifugation (16). Approximately 10 g of yogurt sample was placed into a pre-weighed centrifuge tube and centrifuged at 4000 rpm for 20 min at 4 °C. After centrifugation, the supernatant was carefully decanted and the tube containing the remaining gel was weighed. WHC was calculated using the following formula 2:

$$WHC\ (\%) = \left(1 - \frac{W_o - W}{W_o}\right) * 100 \qquad \text{(Eqn. 2)}$$

Where: W is the weight of sample after centrifugation, W_o is initial weight of sample

Data analysis

Statgraphics Centurion XVI software (USA) was used to analyze the statistical data. Least significant data (LSD) test was used to determine the difference between experimental mean values at the 5 % confidence level ($p = 0.05$). Microsoft Excel software was used for calculation and graph representation.

Results and Discussion

Effects of the investigating factors on bacterial density and fermentation time of yogurt

Starter cultures refer to the microbial strains responsible for initiating fermentation processes and are selected based on the desired end product (24). In yogurt production technology, LAB plays a critical role by fermenting lactose to produce lactic acid, which leads to milk coagulation and the formation of characteristic aroma compounds. The commercial yogurt starter (CYS), which is rich in beneficial bacteria such as *Lactobacillus delbrueckii, Streptococcus thermophilus,* was used as the initial inoculum in the production of yogurt from purple rice and soymilk. The addition of microbial cultures in the form of a starter serves as the basis for ensuring that the products are manufactured through a consistent process, resulting in uniform product quality (25).

The inoculation rate of CYS affects the bacterial density in the propagation medium. An appropriate inoculum level is critical in determining both the quality and cost of the final product (26). The results presented in the table indicate that increasing the inoculation rate of the commercial yogurt starter in the enrichment medium led to a rise in bacterial cell counts to an optimal level, followed by a decline, with significant differences observed at the 99 % confidence level. Specifically, the bacterial population reached 3.21×10^8 cfu/mL after incubating with 0.5 % CYS and continued increasing up to 4.64×10^8 cfu/mL at 1.5 % culture. However, the bacterial density declined to 4.07×10^8 cfu/mL when persisting in adding yogurt starter up to 2 %. An increased inoculation level results in a higher bacterial count within the propagation medium (27). However, under consistent nutrient concentration and medium volume, only the optimal inoculum ratio can yield the maximum biomass.

A low inoculation level prolongs the bacterial lag phase, whereas an excessively high inoculum leads to rapid nutrient depletion, triggering cellular aging (senescence) and a consequent decline in viable cell numbers (28). Bacterial density influences growth rates at excessively high densities, thermal interactions may occur and negatively impacting bacterial growth (29). With a low inoculum, the bacterial population is insufficient to produce antimicrobial compounds, conversely, at the optimal inoculation level, bacterial cells become physiologically active and begin producing antimicrobial compounds as a survival mechanism. Maximum antimicrobial production is correlated with maximum cell biomass (30).

A similar trend was observed in bacterial growth over the incubation period (Table 1). The cell count increased to an optimal value and then declined with extended incubation time. Specifically, the bacterial population in the enrichment medium was 1.57×10^8 cfu/mL after 8 hr of incubation, which rose to 5.41×10^8 cfu/mL at 32 hr. When incubation time was extended beyond 40, bacterial densities went down at 4.89×10^8 cfu/mL. Selecting an appropriate propagation time is crucial to ensure that the microbial population reaches the standard level required for yogurt fermentation while minimizing time and production costs, which is essential for large-scale microbial preparation (26).

During the early propagation phase, bacteria require time to adapt to the new environment, resulting in a slow growth rate. This is followed by active growth where nutrients are utilized for biomass production, leading to a rapid increase in bacterial density until nutrient depletion occurs, causing a decline in viable cell numbers (31). Growth kinetic studies of *L. acidophilus* strains VTCC 12257 demonstrated that the growth curve reached a plateau after 32 hr, possibly due to accumulation of metabolic by-products or nutrient limitation (30). Previous studies have also shown that with constant inoculation levels, prolonged propagation times will result in gradual depletion of nutrients in the medium, leading to cell death, reduced bacterial density and progression into the death phase (26, 32).

Table 1. Effect of the ratio of commercial starter culture and incubation duration on the bacterial density, fermentation time, lactic acid content and water holding capacity of yogurt

The ratio of CYS (%)	Bacterial density (cfu/mL)	Fermentation time (min)
0,5	$3.21 \times 10^{8d*}$	417^a
1,0	4.33×10^{8b}	403^b
1,5	4.64×10^{8a}	373^c
2,0	4.07×10^{8c}	360^d
Level of significance	**	**
Pre-culture incubation time (hr)	**Bacterial density (cfu/mL)**	**Fermentation time (min)**
8	157×10^{8f}	418^a
16	282×10^{8e}	406^b
24	446×10^{8d}	395^c
32	541×10^{8a}	381^d
40	523×10^{8b}	363^e
48	489×10^{8c}	368^f
Level of significance	**	**
Significance level of interaction	**	**

*Average results of three repetitions. Distinct lowercase letters in each column differ statistically significantly, **difference at 1 % significance level; CYS: commercial yogurt starter.

Fermentation capacity is influenced by the proliferation stage of starter cultures. The ratio of CYS affects the fermentation rate and as a result, determines the duration of the process, as presented in Table 1. Generally, the fermentation process would be stopped when the pH of the sample reached 4.6 as the commercial product (10, 33). The fermentation time was shortened as the proportion of starter culture supplementation increased. Similarly, extending the initial incubation time also contributed to a reduction in the overall fermentation duration. A significant difference was observed in the fermentation times among the samples. Specifically, when the proportion of added CYS increased from 0.5 % to 1.5 %, the fermentation time of yogurt decreased from 417 min to 373 min.

At a supplementation level of 2 %, the fermentation time was further reduced to 360 min. Moreover, the duration of the initial activation incubation had a significant impact on the fermentation process. The pH of the yogurt reached 4.6 at 418, 381 and 363 min corresponding to initial activation times of 8 hr, 32 hr and 40 hr, respectively. However, the extension of the incubation time from 40 to 48 hr resulted in a prolonged fermentation time can be explained by the decrease in bacterial count from 5.23×10^8 to 4.89×10^8 cfu/mL (Table 1). A significant correlation was observed between the initial bacterial population and the fermentation duration in plant-based yogurt production (34).

The optimal activation incubation time proliferate bacteria to a high cell density, thereby shortening the fermentation time. A higher bacterial density accelerates the conversion of lactose into lactic acid, leading to a more rapid decrease in the pH of the yogurt and enabling it to reach the target pH in a shorter period. However, if the incubating duration is prolonged excessively, bacterial viability may decline due to nutrient depletion and the accumulation of metabolic byproducts (lactic acid), resulting in a reduced number of viable cells and a slower fermentation rate.

Effect of inoculum ratio and incubation time on yogurt properties

The properties of yogurt samples including lactic acid and water holding capacity were observed after fermentation (Fig. 1). During yogurt fermentation, carbohydrates such as lactose are metabolized into lactic acid by LAB. Therefore, the quality of yogurt can be evaluated based on lactic acid content. The inoculum ratio and the incubation time significantly influence the fermentation kinetics and lactic acid production in yogurt, particularly at the critical point when the pH reaches the standard level of 4.6 (18).

Increasing the inoculum concentration (CYS) from 0.5 % to 1.5 % and 2.0 % significantly reduced the fermentation time, this indicates that a higher initial bacterial population accelerates the lactose-to-lactic acid conversion process (12, 35). As the results, a significant difference was also observed in the amount of lactic acid produced during fermentation. The higher the initial inoculum concentration, the greater the lactic acid production ranged from 0.435 - 0.453 %. Similarly, the duration of pre-culture incubation plays a crucial role, when extending the incubation time from 8 hr to 32 hr and 40 hr shortened the fermentation time. However, when the pre-culture period was prolonged to 48 hr, the fermentation time increased again due to a decline in viable bacterial counts, likely caused by nutrient depletion and the accumulation of metabolic by-products such as lactic acid, which compromise bacterial viability as presented in Table 1 (12). Significantly, the lactic acid concentration at 48 hr of pre-culture did not differ significantly ($p > 0.05$) from that of samples pre-cultured for 32 hr at 0.45 %.

Water holding capacity (WHC) is a critical attribute of yogurt products that can significantly influence both shelf life and sensory acceptability. The WHC of the yogurt samples was well maintained, exceeding 70 %. Previous studies on plant-based yogurt products have reported WHC values ranging from 60 % to 90 % (20, 36). The high WHC observed in the soybean-purple rice yogurt samples are primarily attributed to the elevated starch content derived from rice. Starch acts as an effective stabilizing agent, promoting water retention within the yogurt matrix (37). This effect is further supported by hydrogen bonding and hydrophobic interactions between protein-protein and protein-carbohydrate complexes, which enhance molecular interactions and structural organization in plant-based yogurt, thereby reducing phase separation (8).

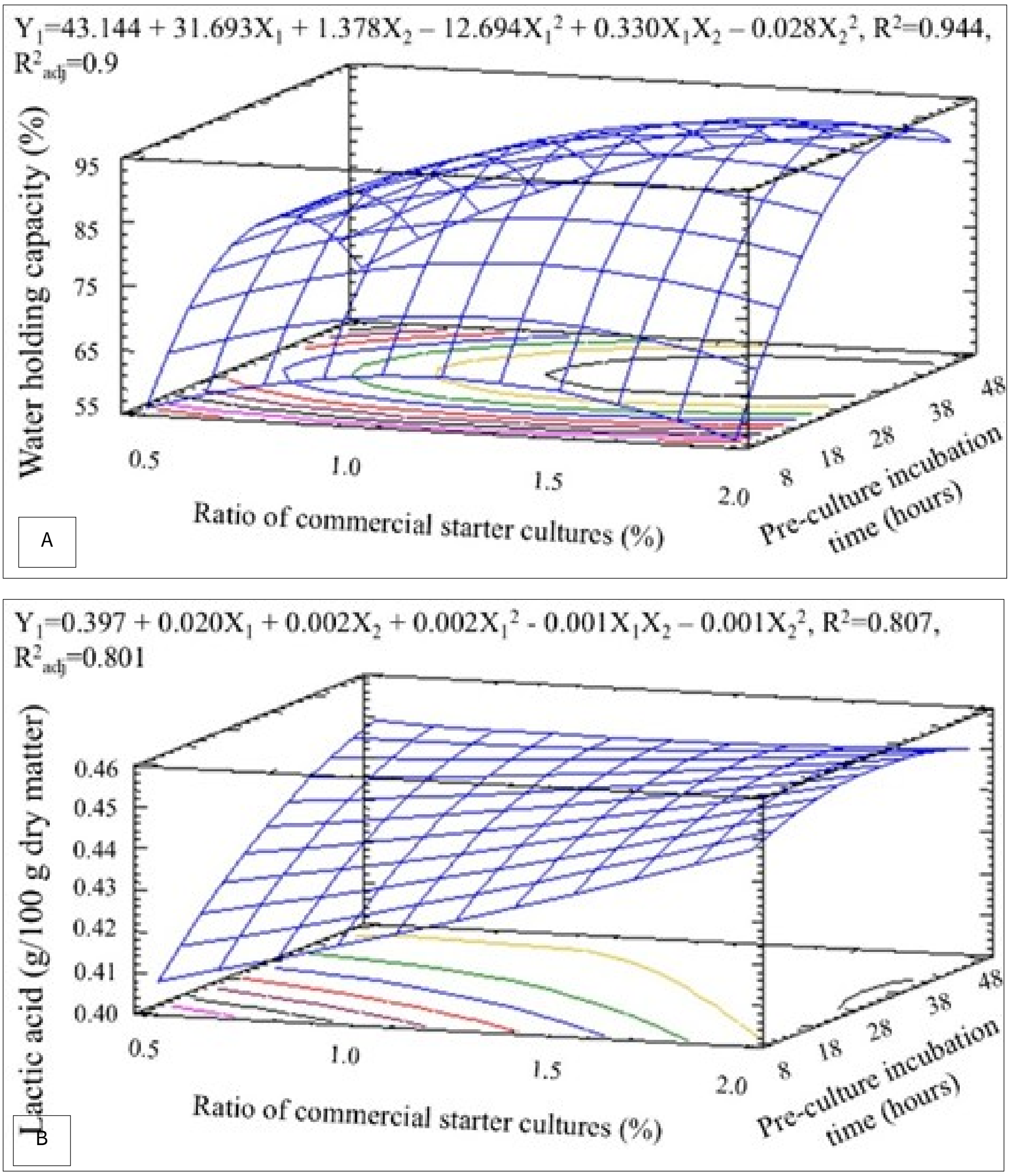

Fig. 1. Response surface graphics demonstrate effects of the ratio of CYS and pre-culture incubating duration on yogurt's properties. (A) lactic acid content (%); (B) water holding capacity (%).

WHC was also found to be closely associated with both the inoculum ratio and the pre-fermentation incubation time, as these factors affect gel structure and lactic acid production. Increasing the inoculum (CYS) level from 0.5 % to 1.5 % resulted in a higher initial bacterial density during the activation phase, which accelerated the fermentation process, led to faster pH reduction, promoted protein coagulation and improved gel strength raising the WHC from 72.03 % to 87.28 %. However, further increasing the inoculum ratio to 2.0 % caused excessively rapid gel formation, potentially leading to an unstable structure and whey separation, thereby slightly reducing WHC to 86.14 %. Similarly, extending the activation time of the starter culture directly influenced bacterial viability and density. At 32 hr, optimal bacterial growth supported uniform fermentation and strong gel formation, resulting in the highest WHC of 88.99 %. Conversely, excessive incubation time (48 hr) led to reduced bacterial viability due to nutrient depletion and accumulation of metabolic byproducts such as lactic acid, weakening the gel structure and decreasing WHC to 80.78 %. Through observing the final products, the obvious effects of the inoculum ratio of commercial starter culture and pre-culture incubation time are demonstrated in Fig. 2. Particularly, at the same pre-culture incubating duration, varying inoculum ratios presented different yogurt surface properties. Conversely, with a fixed inoculum ratio, altering in incubation time also led to noticeable changes in yogurt texture. As shown in Fig. 2A, sample was created with a 1.0 % inoculum pre-cultured for 32 hr had a less smooth surface compared to the yogurt with a 1.5 % inoculum (Fig. 2B). At this 1.5 % ratio, the yogurt exhibited a smooth surface without phase separation when incubation for 32 hr. However, when shortening the pre-culture incubation duration to 8 hr, significant syneresis was observed in the sample (Fig. 2C).

Fig. 2. Purple rice yogurt samples were observed at different conditions and pre-culture incubation durations (A) 1.0 % of CYS for 32 hr incubation; (B) 1.5 % of CYS for 32 hr incubation and (C) 1.5 % of CYS for 8 hr incubation.

Conclusion

The study identified optimal conditions for producing plant-based yogurt using soybean and purple rice. A 1.5 % commercial yogurt starter combined with a 32 hr pre-culture activation period provided a balance outcome in terms of microbial viability, fermentation efficiency, lactic acid production and gel structure. Under this setup, the bacterial density was counted at 5.81 × 108 (cfu/mL) and fermentation time was significantly shortened to 360 min. The WHC reached its peak at 94.66 %, indicating a stable gel with minimal syneresis and improved sensory quality. Therefore, this study demonstrated optimal conditions for the pre-fermentation stage, which could enhance sustainability and reduce production costs, paving the way for further research in this area, especially on fermentation processes and product refinement. Future studies may focus on the development and enhancement of bioactive compounds in yogurt alternative derived from ST purple brown rice.

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Authors' contributions

NTNG designed, carried out the experiment, analysed data, wrote, reviewed and edited the manuscript. NDT, HTNH and DVT carried out the experiment and analysed the data. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

The authors confirm that generative Artificial Intelligence (AI) or AI-assisted technologies were used only to a limited extent, during the writing process, primarily for improving language clarity and grammar. All scientific content, data interpretation and conclusions are the original work of the authors. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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