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Some Factors Affecting Free and Immobilized Amylase Activity

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ABSTRACT

In our study, we prepared chitosan beads and immobilized bacterial amylase enzyme on these beads; Parameters such as comparing the optimization conditions (pH, pH stability, temperature, thermal stability, substrate concentration) of free bacterial amylase and amylase immobilized on chitosan beads and investigating the effect of immobilization in its usage areas are included. The immobilized enzyme showed better activity at both lower and higher pH levels compared to the free enzyme. When considering pH stability, the results were consistent with the pH activity values. In other words, both free and immobilized amylase exhibited the highest activity at the same pH levels. However, the free amylase showed less stability at low and high pH levels compared to the immobilized amylase. In terms of temperature-dependent activity, it was found that both free and immobilized enzymes showed the best activity at the same temperature. However, the immobilized enzyme showed activity at higher temperatures than the free enzyme. Moreover, when studying the effect of substrate concentration on free and immobilized enzymes, both types of enzymes exhibited the best activity in the same concentration of starch solution. In this study, factors affecting immobilized and free amylase activity were examined.

Keywords: Chitosan, Amylase, Enzyme immobilization.

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Introduction

Enzymes, which are biological catalysts in protein structures, ensure that biological and chemical reactions in living organisms proceed under appropriate and efficient conditions. Enzymes can function outside their natural environment if suitable conditions are provided. They have been successfully utilized in the fields of economy, health, food, cosmetics, industry, and many other everyday applications. The cost-effectiveness of enzyme-catalyzed reactions compared to chemical methods is one of the key reasons for the widespread integration of enzymes into our daily lives [1]. However, its distribution in areas of use is shaped by the activity of enzymes. Some factors affect enzyme activity. Some of these are explained and listed below;

Enzymes show highest activity in a certain pH range. Outside this optimum pH, the activity of enzymes decreases and the reaction rate decreases. The three-dimensional structure of enzymes depends on the pH level. Incorrect pH can cause enzymes to denature (deteriorate their structure). pH can change the binding ability of the substrate by affecting the charge distribution in the active site of the enzyme. This affects the formation of the enzyme-substrate complex. The ionization state of amino acids in the active sites of enzymes depends on pH. This may affect the functionality of the enzymes. Enzymes have evolved to function at various pH levels based on their environment, such as stomach acid or intestinal pH. For these reasons, pH is a critical factor for the activity and functionality of enzymes [2-4].

Enzyme stability involves the balance between the intramolecular interactions of functional groups and their interactions with the environment. These conditions often differ significantly from natural sources of enzymes, such as the presence of organic solvents, unfavorable pH and high ionic strength. This can lead to premature denaturation of enzymes and loss of their activity. For the successful use of enzymes in free or immobilized form, a comprehensive understanding of their long-term stability under various conditions is critical [5-7].

Enzymes show highest activity in certain temperature ranges. At optimum temperature, the reaction rates of enzymes increase. High temperatures can disrupt the structure of enzymes and lead to denaturation. This results in degradation of the enzymes' active sites and loss of functionality. As the temperature increases, the kinetic energy of the enzyme and substrate molecules also increases. This leads to more collisions of molecules, thus increasing the reaction rate. However, after a certain point, this increase in speed can result in denaturation [8,9].

Thermal stability refers to the resistance of enzymes to high temperatures. Some enzymes, especially those from thermophilic organisms, can remain stable at high temperatures, providing an advantage in industrial applications. Enzymes function in different temperature ranges depending on the organisms and environmental conditions in which they are found. Thermal stability makes it possible for them to adapt to these conditions [9,10].

A lot of industrial processes use water-based solutions, and sometimes free enzymes are used as catalysts. When free enzymes are used, it's difficult to recover them from the environment until they have finished their job. This, along with other factors like the contamination of the reaction medium with inhibitors, the limited reusability, and the challenge of removing the enzyme at the right time, makes enzymes expensive and less versatile as catalysts. As a result, using enzymes can drive up costs in industrial settings [11].

The interest in studying enzyme immobilization has grown to tackle various challenges, find effective solutions, and enhance the cost-efficiency and applicability of enzymes, especially in industrial contexts. These studies have been ongoing for almost fifty years. Research on immobilized enzymes has highlighted the role of free enzymes as a bridge connecting immobilized enzymes to their intended applications. However, the primary emphasis has been on the use of immobilized enzymes themselves. In the process of enzyme immobilization, various organic and inorganic substances are utilized as support materials, including chitosan, chitin, cellulose, silica, agarose, phenolic resin, polymers, and bone [12-16]. Many studies in the literature have characterized enzymes by immobilizing them on various supports, with chitosan being one of the most commonly used materials. A comprehensive review outlined the methods for immobilizing catalase enzymes using a range of natural and synthetic polymers, inorganic materials, composite supports, and hybrids. This review emphasized the significance of these immobilization methods in the context of bioreactors and biosensors [17]. Additionally, research has focused on enzyme immobilization and the determination of immobilization conditions using chitosan, a natural polymer derived from the deacetylation of chitin [18,20]. Immobilized enzymes present several advantages compared to free enzymes. Firstly, they can be easily separated from the reaction environment once the reaction is complete, which prevents contamination of the final product. In contrast, free enzymes remain in the reaction environment after the process, potentially affecting the product's quality. Moreover, immobilized enzymes tend to be more resistant to environmental factors such as changes in pH and temperature, making them more stable and reusable. They can also be utilized in continuous processes, allowing for better control over product production and reducing enzymatic degradation. Overall, immobilized enzymes provide a promising solution for continuous multi-step reactions [19,20].

In our study, we utilized chitosan as a support material. Chitosan is a non-toxic and highly biocompatible polymer that is widely used in the biomedical field. Like alginate, chitosan is a biopolymer that can be metabolized in vivo and possesses antitumor properties, as well as the ability to form gels. Additionally, it is effective for treatment due to its bacteriostatic and fungistatic characteristics. Therefore, chitosan and chitin are essential biopolymers for various applications due to their biocompatibility, biological metabolism, adsorption capabilities, and gel-forming properties [20].

In our study, we utilized the bacterial enzyme amylase. This enzyme was selected due to its significant market share, which accounts for nearly half of the industrial enzyme market. Additionally, certain microorganisms produce various enzymes internally to adapt to their environment and then secrete these enzymes through their cell walls. Amylase is one such enzyme, playing a crucial role in breaking down natural raw materials, such as starch. This enzyme, produced through modern methods, has great potential for widespread industrial application and is particularly economically advantageous in a country like Turkey, where there is an abundance of fermentable agricultural residues [21,26].

The bacterial origin of the enzyme contributes to its efficiency and cost-effectiveness, providing it with a significant advantage. Achieving a balance between high efficiency and low cost is challenging for enzymes. Amylase (EC 3.2.1.1) is utilized in various industrial processes, sourced from foods, textiles, paper, and solid waste. This enzyme plays a crucial role in the transformation of starch molecules by hydrolyzing them in the presence of water, resulting in diverse products. Specifically, amylase (1-4-α-glucan-glucanohydrolase) belongs to the hydrolase group, which typically cleaves glycogen and starch-like polysaccharides at alpha-1-4 glycosidic bonds, thereby producing oligosaccharides of various lengths. This enzyme is widely present in many plants, animals, bacteria, and fungi that utilize polysaccharides, making it the primary form of amylase found in humans and other mammals [22].

Amylases and glycoside hydrolases are classified into 13 families. Amylase is a highly valuable industrial enzyme, commonly used in the fruit juice, food, detergent production, pharmaceutical, and textile industries [23-25]. It is also found in seeds that contain starch as a food reserve and is secreted by various fungi.

Material and Method

Chemical Substances Used

Amylase (EC 3.2.1.1) (Sigma- Aldrich), Soluble Starch, (Sigma-Aldrich), Chitosan (Sigma-Aldrich), Sodium Hydroxide- NAOH (Sigma-Aldrich), K/N tartrate (Sigma-Aldrich), Disodium Hydrogen Phosphate Dihydrate- Na_2HPO_4 (ISO-LAB), Sodium Dihydrogen Phosphate Dihydrate- NaH_2PO_4 (ISO-LAB), Boric Acid-H_3BO_3(ISO-LAB), Potassium Chloride- KCl(ISO-LAB), Citric Acid- $C_6H_8O_7$(ISO-LAB), Sodium Citrate, $Na_3C_6H_5O_7$(Merck), DNS (Merck),

Glyoxal (Merck), Glacial Acetic Acid (Sigma-Aldrich), All chemicals used in the study were of analytical purity (≥98%) and double distilled water was used in all stages of the experiments.

Preparation of Chitosan Solution: 3 g of chitosan was suspended in 99 mL of distilled water with a magnetic stirrer. 1 mL of glacial acetic acid was added and stirred for 3 hours at room temperature, and insoluble particles

were separated through a filter. This solution can be used for 1 week at room temperature.

Preparation of Crosslinker Solution: Dissolve 10 g of glyoxal in 250 mL of distilled water. It is dissolved by heating at 80ºC for 10 minutes and cooled at room temperature. This solution is mixed with an equal volume (%4 w/v) of tetrasodium pyrophosphate (pH 8). The solution was prepared fresh and used on the same day.

Preparation of Chitosan Beads

The filtered and rested chitosan solution, which was free of bubbles, was carefully filled into a syringe and slowly dropped into the cross-linker solution one by one (5 mL of cross-linker was dropped into 1 mL of chitosan). The beads were left to mature in this solution for 30 minutes, externally cooled with ice. Then, it was washed 3 times with 0.05 M pH 7 phosphate buffer.

Strengthening of Chitosan Beads with Glutaraldehyde

Freshly prepared beads were incubated in 0.05% (w/v) glutaraldehyde solution for 1 hour. The beads that turned Orange-Brown were washed several times with cold 0.05 M pH 7 phosphate buffer.

Amylase Immobilization into Chitosan Beads

2 mL of stock amylase solution prepared in pH: 7 phosphate buffer at a concentration of 500 ppm was taken. 10 chitosan beads were added and incubated in a shaking incubator for 120 minutes. It was then washed three times with 50 mM phosphate buffer pH:7 to remove unbound enzyme.

$$Q = (C_o - C_1) \backslash m \; X \; Vt \quad (1)$$

The amount of protein bound to the immobilized support was determined by protein determination. For this purpose, the Bradford (1976) protein determination method was used. The amount of bound protein (Q) was calculated by subtracting the amount of protein remaining after immobilization (C1) from the initial amount of protein (Co), dividing by the mass of beads used (m) and multiplying by the total solution volume (Vt) [27].

Investigation of the Activity of Immobilized and Free Amylase in Different Parameters

Amylase activity was determined spectrophotometrically using Bernfeld's partially modified 3,5-dinitrosalicylic acid (DNS) method [7]. 1% soluble starch prepared in 0.05 M pH 7.0 Phosphate buffer was used as the substrate. The components of the standard experimental environment are given in Table 1. and Table 2. Enzyme activity was measured at 530 nm under standard experimental conditions. The amount of reducing sugar formed was calculated from the standard maltose curve. Enzyme activity and specific activity were calculated according to the equations given below.

DNS solution: It was prepared with a ratio of 10g DNS, 16g NaOH, and 300g K/H tartrate (g/L).

1% Starch solution: Weighed 0.1 g of starch, dissolved in 10 mL of 0.05 M phosphate buffer. Heating was applied to dissolve the starch.

$$Volume\ Activity = \frac{Maltose\ (\mu M)}{\text{Incubation time (dk)x Enzyme Amount (mL)}} = \left({}^{U}/_{mL}\right) \quad (2)$$

$$Specific\ Activity = \frac{\text{Volume Activity } \left({}^{U}/_{mL}\right)}{\text{mg Protein/ mL}} = \left({}^{U}/_{mg\ Protein}\right) \quad (3)$$

The effects of pH, pH stability, temperature and thermal stability on the activity of immobilized and free enzymes were investigated.

Table 1. Free Enzyme Experimental Environment [7].

Sample Tube	Blind Tube
400µL starch	400µL starch
400µL enzyme	-
55°C 20 min. Incubation	55°C 20 min. Incubation
200µL DNS	200µL DNS
-	400µL enzyme
2 min. Cold water bath	2 min. Cold water bath
5 min. Boiling water bath	5 min. Boiling water bath
3 min. Cold water bath	3min. Cold water bath
2000µL distilled water	2000µL distilled water

Table 2. Immobilized Enzyme Experimental Environment [7].

Sample Tube	Blind Tube
400µL starch	400µL starch
10 beads soaked in enzyme	10 empty (non-immobilized) beads
55◦c 20 min. Incubation	55◦c 20 min Incbation
200µL DNS	200µL DNS
2min. cold water bath	2min. cold water bath
5min. Boiling water bath	5min. Boiling water bath
3min. Cold water bath	3min. Cold water bath
2000µL distilled water	2000µL distilled water

Effect of pH on Immobilized and Free Amylase Enzyme Activity

To determine the optimum pH of free and immobilized amylase, measurements were made using substrate solutions prepared in buffers at different pHs (pH: 4, 5, 6, 7, 8, 9) at the optimum temperature (55 ℃) using the experimental environment described in Table 1. and Table 2. Activity calculations were made and graphs were drawn.

Effect of pH Stability on Immobilized and Free Amylase Enzyme Activity

Free and immobilized amylase were incubated for 1 hour at different pH (pH: 4, 5, 6, 7, 8, 9) and measurements were taken using the experimental environment specified in Table 1. and Table 2. Activity calculations were made and graphs were drawn.

Effect of Temperature on Immobilized and Free Amylase Enzyme Activity

Temperature-dependent activity analysis of free and immobilized enzyme was performed. The construction phase of this experiment is as follows: Activity was measured and graphs were drawn under optimal conditions, provided that the incubation time and other steps in the 3rd step (20 min incubation at 55°C) in Table 1. and Table 2. remained constant and the temperature was changed (25- 35- 45- 55- 65- 75°C).

Effect of Thermal Stability on Immobilized and Free Amylase Enzyme Activity

Free and immobilized amylase were incubated at different temperatures (25- 35- 45- 55- 65- 75°C) for 1 hour before the experimental environment. After incubation, activities under optimal conditions were measured, calculated and graphed.

Results and Discussion

Effect of pH on Immobilized and Free Amylase Enzyme Activity

As a result of this experiment, free amylase showed 100% activity at pH: 7, while immobilized amylase showed 100% activity at pH 9. While the free enzyme was denatured at pH: 4, on the contrary, the immobilized enzyme showed activity at the same pH value. All values are shown in Table 3. These values are shown on the graph in Figure 1.

Table 3. Activity% Values of Free and Immobilized Amylase at Different pH

pH Value	Free Amylase Activity %	Immobilized Amylase Activity %
4	0	24,68
5	12,65	34,92
6	55,83	61,24
7	100,00	78,74
8	97,99	97,95
9	92,97	100,00

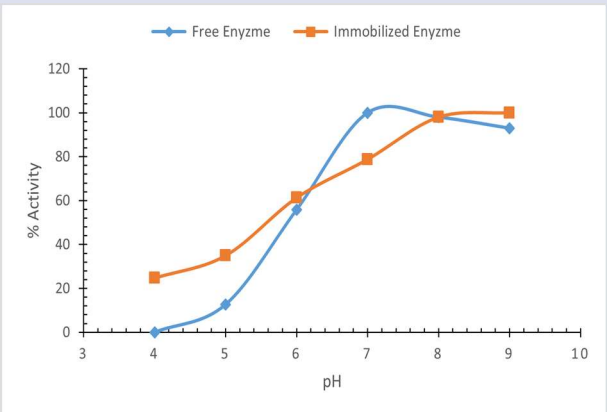

Figure 1. pH Value versus Activity % Value of Free and Immobilized Amylase

Effect of pH Stability on Immobilized and Free Amylase Enzyme Activity

While free amylase was found to have the highest stability at pH 7, immobilized amylase was found to have the highest stability at pH 9. Free amylase was not stable at pH: 4, immobilized amylase, on the contrary, showed low stability at pH: 4. The pH stability of the immobilized enzyme is higher than the stability of the free enzyme. All values are shown in Table 4. These values are shown on the graph in Figure 2.

Table 4. Activity % Values of Free and Immobilized Amylase at Different pHs

pH Value	Free Activity- Stability %	Immobilized Activity- Stability %
4	0	27,48
5	6,46	38,92
6	54,96	56,32
7	100,00	81,84
8	95,38	95,14
9	73,44	100,00

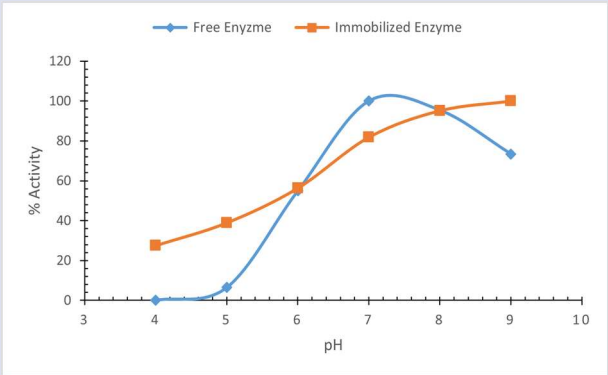

Figure 2. pH value versus Activity % value of free and immobilized amylase

Effect of Temperature on Immobilized and Free Amylase Enzyme Activity

When the % activity values of free and immobilized amylase depending on temperature were examined, it was observed that both enzymes reached 100% activity at 55°C. Both enzymes showed activity at all temperatures, most of which were different from each other. All values are shown in Table 5. The figure covering these data is drawn in Figure 3.

Table 5. Activity % Values of Free and Immobilized Amylase at Different Temperatures

Temperature °C	Free Amylase Activity %	Immobilized Amylase Activity %
25	38,45	46,13
35	66,80	50,93
45	82,00	66,72
55	100,00	100,00
65	97,93	97,72
75	97,237	94,23

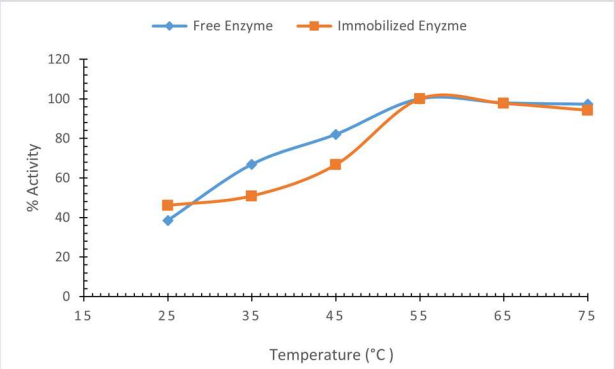

Figure 3. Temperature value versus Activity % value of free and immobilized amylase

The optimum temperature was found to be the same 55°C for both enzymes.

Effect of Thermal Stability on Immobilized and Free Amylase Enzyme Activity

After free and immobilized amylase were incubated for 1 hour under known optimum conditions and at different temperatures, activity measurements were made under optimal conditions. A graph of % activity versus temperature was drawn. As a result, both immobilized enzyme and free enzyme showed the best stability between 55°C and 75°C. Both forms of the enzyme showed stability at all temperatures. All values are shown in Table 6. These values are shown on the graph in Figure 4.

Table 6. Activity-Stability % Values of Free and Immobilized Amylase at Different Temperatures

Temperature ºC	Free Activity- Stability %	Immobilized Amylase Activity- Stability %
25	27,76886	35,55369
35	51,84591	47,61008
45	73,51525	75,69218
55	100	93,26022
65	98,39486	97,30234
75	98,39486	100

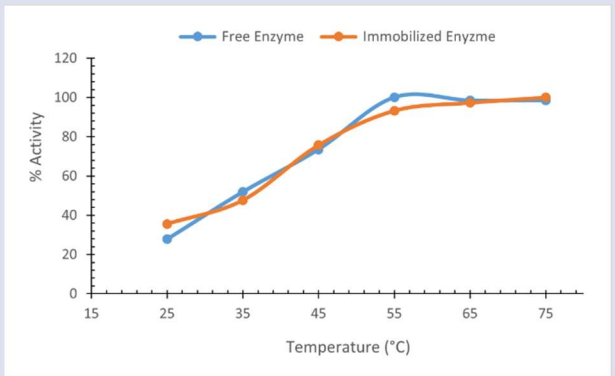

Figure 4. Temperature value of free and immobilized amylase versus Activity-Stability % value

Conclusion

Experiments were performed using different pHs to investigate the effect of pH and pH stability on free amylase and immobilized amylase activity. The optimum pH of the free amylase enzyme was 7, and the optimum pH of the immobilized amylase was 9. Both enzyme forms showed maximum stability at their respective optimum pHs. The variation of maximum activity values with pH is shown in Figure 1 and Figure 2. These figures show a decrease in the maximum activity of free amylase enzyme in acidic and basic regions. One factor that most affects enzymatic reaction rates is the pH of the environment. This enzyme, which has electrolyte properties and therefore enzyme activity is affected by pH, also contains acidic and basic groups in its molecules such as substrate and coenzyme. For this reason, enzymes, substrates, and coenzymes will inevitably be affected by pH. The resulting pH changes affect the stability of the enzyme-substrate complex. Stability and reaction speed are directly proportional. The more stable an enzyme+ substrate complex, the closer the reaction rate is to a maximum. Optimum pH is the pH value at which the enzyme is most active. The enzyme draws a pH curve in the form of a bell curve, the activity and reaction rate of the enzyme irreversibly decreases to zero at the end of both sides of the pH and the enzyme denatures. The optimum pH for enzymes varies depending on various conditions. Factors that cause changes in optimal pH include the source of the enzyme, the type and concentration of substrate on which the enzyme is used, temperature, type of cofactor, and time. The enzyme is not stable at all pH values. Every enzyme has a pH at which it maintains its stability [16,17]. On the other hand, different interactions may occur between enzymes and polymeric support materials. It is known that forces affect pH [18-20].

In our study, amylase was immobilized on chitosan beads as solid support material. Thus, the enzyme was made usable at higher pH values. Because the immobilized enzyme is active at every pH studied, it provides advantages in every field where it is used.

Experiments were carried out at different temperatures to investigate the thermal stability of free and immobilized amylase activity. The optimum temperature of both enzyme forms was determined as 55°C. In thermal stability, this temperature remained at 55°C for the free enzyme, while it was observed at 75°C for the immobilized enzyme. The change of maximum activity-stability values with temperature is shown in Figure 3 and Figure 4. Looking at these figures, a decrease in the maximum activity of free amylase enzyme was observed in regions where the temperature was low and high.

The activities of the enzymes in both forms at their optimum temperatures were accepted as 100% and the other percentages were calculated according to this value. The free and immobilized enzyme showed activity at all temperatures. In free enzyme, when the temperature is lowered or higher than 55°C, the activity decreases, and

when the temperature rises above a certain temperature, the activity of the enzyme drops to zero and the enzyme is irreversibly denatured. However, at low temperatures, when the enzyme freezes or something similar, the enzyme activity reversibly drops to zero, that is, when the temperature is increased to normal conditions again, the enzyme will show activity again, and this is valid for both immobilized and free amylase. The temperature stability of immobilized enzymes is one of the most important reasons for using immobilized enzymes. Enzymes that are immobilized through cross-linking are more resistant to high temperatures compared to free enzymes. This ensures that the immobilized enzyme has constant activity for a certain period during use, facilitating control of the process.

The conformational flexibility of enzymes is affected by immobilization. Immobilization reduces the flexibility of the enzyme and increases its rigidity. This usually manifests itself as increased stability against increasing temperatures. Many studies have shown that the temperature stability of immobilized enzymes, where immobilization reduces their conformational flexibility, is higher than that of free enzymes [21]. Generally, a 10°C increase in temperature approximately doubles the reaction rate. The same situation is observed in biochemical reactions where enzymes in protein structures begin to denature at certain temperatures (above 50 °C) [22]. As the temperature increases, first the tertiary structure and then the secondary structure (α-helical structure) of the enzyme molecule collapse. The active site of the enzyme is also affected by these events and the enzyme loses its activity. When all these are evaluated; The fact that the immobilized enzyme is more stable at higher temperatures than the free enzyme allows this enzyme to be used at high temperatures in its areas of use, thus reducing the cost when considered with pH. This allows it to be more preferred in areas of use.

The narrow parameter ranges of the free enzyme cause the use of the enzyme to be costly. In the experiments, it is seen that the parameter ranges expand with immobilization. This result supports that, with immobilization, the enzyme can now be used under more challenging operating conditions. The immobilization studies were carried out successfully. This study, which can be considered at the initial level in the literature, has been a step towards increasing working conditions to further levels by expanding them.

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