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# MUTATION STUDIES ON FUNGAL GLUCOAMYLASE: A REVIEW

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## ABSTRACT

*The potential of using fungi as source of industrially important and relevant enzymes has stimulated interest in exploration of enzymatic activities. Starch is one of the most important naturally occurring polymers and appears to be the cheapest future raw material of alcohol industry. This paper proposes to discuss some recent work on the strain improvement strategies highlighting the mutagenesis for the increased production of glucoamylase and its increasing role in biotechnological process, which has made a great impact on the enzyme industry. Glucoamylase (α -1,4glucan glucohydrolase, amyloglucosidase) is of great importance to the fermentation and food industries for saccharification of starch and other related oligosaccharides. The fungal glucoamylase is used effectively in various industries like pharmaceutical industry, food industry, baking industry etc. In view of biotechnological importance of fungal glucoamylase, the review reveals the various aspects of improvement of industrial potential of enzymes highlighting mutation studies.*

***KEY WORDS***

*Starch, strain improvement, mutagenesis, fungal glucoamylase, industrial potential*

## INTRODUCTION

Microorganisms have been exploited in the production of industrial chemicals. The different industries use different organisms in fermentation processes to yield a variety of organic acids, enzymes, chemicals, pharmaceuticals, biomass, beverages etc. Microbial technology is not only involved in producing high value industrial products but also involved in processing industrial wastes to generate useful by products and to keep environment clean. Microbial production of primary metabolites contributes significantly to the quality of life. Through fermentation, microorganism growing on inexpensive carbon sources can produce valuable products such as enzyme, amino acid, nucleotides organic acids and vitamins which can be added to food to enhance its flavor or increase or its nutritive value. Starchy biomass appears to be the cheapest future raw material of alcohol industry in view of rising cost and deteriorating quality of fermentable molasses [1]. Thus, significant interests exist in research on the amylolytic enzymes as the conventional production of ethanol from starch [2].

Enzymes responsible for degradation of starch and related saccharides are produced either by prokaryotic or eukaryotic organisms. Starch is not hydrolysed completely by a single enzyme

but is attacked by variety of enzymes, generally called amylolytic enzymes, produced by a large number of microorganisms including bacteria, fungi, yeast, algae and actinomycetes which have the ability of utilizing starch as energy and carbon source [3]. Amylolytic enzymes of microbial origin are divided into exo-acting, endo-acting, debranching and cyclodextrin producing enzymes [4].

**NEED FOR STRAIN IMPROVEMENT**

The strain improvement is the process of improvement and manipulation of microbial strains for the enhancement of metabolic capacities for biotechnological applications [5]. The yield of microbial enzymes can be increased by mounting a suitable medium for fermentation, refining the fermentation process and strain improvement for higher production. This is done in order to minimize the production cost. Since, the microorganisms produce array of valuable products but the quantity produced is beneficial for them only, therefore overproduction of metabolites rarely occurs. There is tremendous contribution of genetics and genetic engineering to the overproduction of microbial products, which can be achieved through mutation, selection/screening and application of recombinant DNA technology. These mainly focus at the increase in fermentation productivity, cost reduction and economical benefits since the wild strains produce low metabolite concentration. The industrial microorganism should have genetic stability, efficient production of biosynthetic metabolic products, non pathogenic, safe, harmless, easily accessible for genetic manipulation, non toxic, easily harvested from fermentation and low cost [6]. The genetic manipulation of fungi is done to increase the production of desired product, to increase the tolerance of a specific factor and to make them capable of expressing a recombinant protein.

The use of glucoamylase for the production of various food grade metabolites has been increased exponentially. Several mutational strategies are used to improve the production of enzyme and traditional mutagenesis is important tool for the generation of potential hyper productive mutants [2]. The strategies for improvement of strains depend on the fermentation process and nature of the products [7]. The common example of strain improvement is the production of antibiotic from a soil isolate. The process of random mutagenesis is done for strain improvement of antibiotics. Further, the empirical approach was used for strain improvement. The mutagens like nitrososguanidine, UV, caffeine are used in empirical approach which induces single mutation in DNA of producer organism; some of these mutations are harmful which affect the growth of organism leading to ceasation of colony. These colonies are then plated on agar media to access for strain improvement. This process is very common in pharmaceutical industries for the production of antibiotics [7].

**FUNGI AS CELL FACTORIES FOR GLUCOAMYLASE PRODUCTION**

Microorganisms are widely exploited as cell factories in the food and beverage industries worldwide. The food biotechnologies mainly aims at improved food production but are also dependent on the demand of consumer for safe, natural, fresh tasteful and convenient products [8]. This led the research to mainly focus on the exploitation of potential application of microorganism in human nutrition and animal feeding. The glucoamylase produced by micro-organisms has potential for the hydrolysis of starch into various sugars. The microorganisms have shorter life span and the enzyme of microbial origin can be isolated easily and their characteristics can be manipulated by genetic engineering and

biotechnology techniques. Now the myco-technology took commendable consign in biotechnology as the Association of manufacturers of Fermentation Enzyme products (AMFEP) shows that most enzymes are produced by filamentous fungi [2].

Fungi are mostly employed to produce industrially important glucoamylase [9]. Many fungal species are capable of producing glucoamylase under different fermentation conditions and techniques [10]. Glucoamylase occur almost exclusively in fungi and the industrial focus has been on Glucoamylase from *Aspergillus niger* [11] and *Rhizopus oryzae* [12]. Also *Aspergillus awamori* [13] and *Aspergillus oryzae* [14] still belong to the most intensively studied fungus and have been considered the most important for commercial production in industries.

Enzyme biotechnology aims at the production of enzymes by using various strains of bacteria and fungi in submerged and solid state fermentation [15]. The filamentous fungi are mostly preferred for commercial production of enzymes because the enzymes produced by these fungi are more efficient as compared to those obtained from yeast and bacteria [16]. In recent years, the glucoamylase production by solid state fermentation is gaining interest because of low cost and increased productivity and prospects of using a wide range of agro-industrial residues as substrates [17, 18]. The carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are very expensive for the commercial production of glucoamylase. Various agricultural byproducts like wheat bran, rice bran, rice husk, sugarcane bagasse, oat bran, cotton seed meal are available for the conversion to products of economic importance [19].

**Table 1: Physicochemical Properties of Fungal Glucoamylase**

| S. No. | Source | No. of forms | Molecular weight (Kda) | pH optimum | Temperature optimum | Reference |
|---|---|---|---|---|---|---|
| 1. | *Scytalidium thermophilum* | - | 86.00 | 6.50 | 60 | [21] |
| 2. | *Aspergillus oryzae* | 3 | 38-76 | 4.5 | 50-60 | [20] |
| 3. | *Aspergillus awamori* | 1 | 83.7 | 4.5 | 60 | [22] |
| 4. | *Aspergillus niger* | 4 | 61-112 | 4.4 | 60 | [23] |
| 5. | *Aspergillus niger* | 2 | 74-96 | 4.2,4.5 | 60, 65 | [24] |
| 6. | *Aspergillus awamori var kawachii* | 3 | 57-90 | 3.8-4.5 | - | [25] |
| 7. | *Aspergillus terreus* | 1 | 70 | 5 | 60 | [26] |
| 8. | *Aspergillus saitoi* | 1 | 90 | 4.5 | - | [27] |
| 9. | *Rhizopus* sp. | 3 | 58.6-74 | 4.5-5 | - | [28] |
| 10. | *Acremonium* sp. YT 78 | - | 74 | 5.0 | 50 | [29] |

Glucoamylases (GAs) also known as 1,4-β-D-Glucan glucohydrolase, amyloglucosidase or gamma amylase (EC 3.2.1.3) are exo-amylases as they act on the polymers from the non reducing chain ends and release β- D glucose residues [3]. These hydrolyze α -1,4 glycosidic

linkages in raw or soluble starches and related oligosaccharides, producing β-glucose by inversion of the anomeric configuration. In addition, they also hydrolyze α -1,6 glycosidic linkages of starch but at a lower rate [20]. This enzyme catalyses the hydrolysis of glucose based polymers such as starch to release glucose and α dextrin with less polymerization degree. Some of the physicochemical properties of fungal glucoamylase are enlisted in Table 1.

**MOLECULAR STUDY FOR INCREASED GLUCOAMYLASE PRODUCTION**

Molecular studies are done either for increasing the product yield or for altering the properties of the product. Increased yield of enzyme may be achieved by optimizing the culture medium and growth conditions but this approach is limited to the ability of organism to synthesize the product. Strain improvement is done to reduce cost by increasing productivity or by reducing manufacturing cost so it is plays a vital role in fermentation industries [6]. The mutational strategies are used to improve the enzyme productivity**.** The fermentation technology is dependent on the superior strain development by mutagenesis, random screening procedures and physical and chemical environment optimization [30]. Genetic modification studies over the past 10 years have contributed a great deal to understand the thermo-stability and active site mechanism for industrial improvement. Most of the wild strains which have potential use in industrial fermentation processes are subjected to industrial strain improvement to make fermentation economical [31].

The strain improvement of industrially important microorganisms is carried out by a variety of methods such as mutations, protoplast fusion, recombinant DNA technology and gene cloning [32] as shown in (Fig. 1). Among these, random mutagenesis and protoplast fusion are the simpler and commonly used techniques. The mutagenic processes involve physical, chemical and site directed mutagenesis for strain improvement. The over production of industrial products by strain improvement has been considered in commercial fermentation process [32, 33].

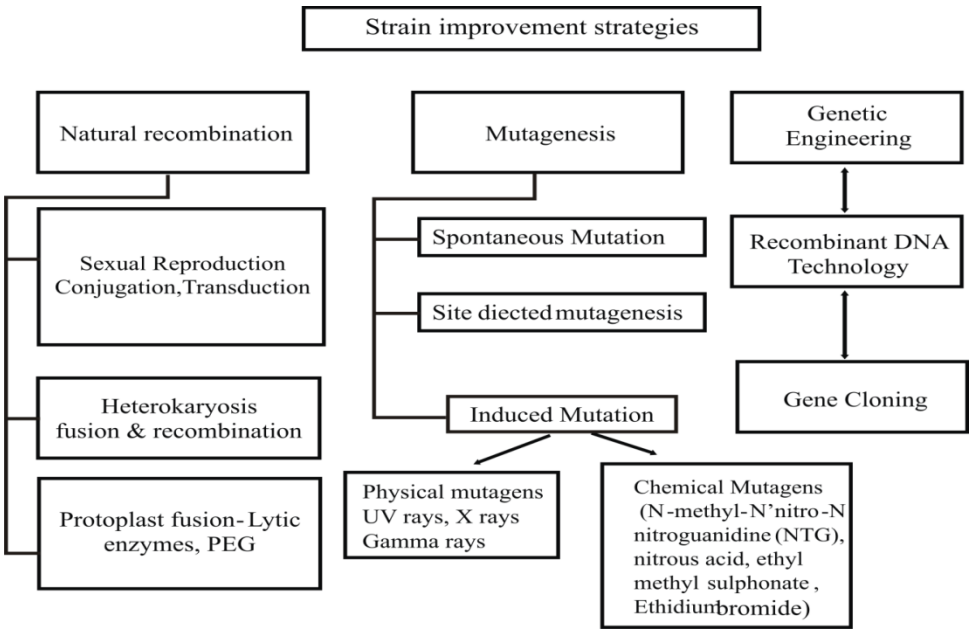


**Fig. 1 Overview of Strain Improvement**

**Natural Recombination:**

This occurs in bacteria by the transfer of the genetic material by conjugation, transduction and transformation [6]. The genetic recombination involves union of two different genotypes to form new genotypes. Then it involves the screening and selection of specific strains for industrial fermentation process.

The process of Transformation in fungi was first reported by Mishra and Tatum, [34] who demonstrated increased recovery of inositol independent transformants. This technology offers the possibility of altering the characteristics with surgical precision, modification of an existing property, introduction of new characteristics without adversely affecting other desirable properties or elimination of unwanted trait.

The recombination by Protoplast fusion is a versatile tool for genetic manipulation and breeding in industrial microorganisms. Protoplast fusion has been demonstrated as an efficient way to induce heterokaryon formation and recombination with high frequency [35, 36]. Protoplast fusion in some industrially important microorganisms includes *Streptomyces* spp. [37], Filamentous fungi [38], Yeasts [39]. Since the development of enzyme system able to remove the cell wall of both single celled and filamentous fungi, protoplasts have been the source of wall biogenesis and repair, physiology and biochemistry. With the advent of fusion techniques, protoplasts have been used to bypass many natural barriers to cross breeding in fungi.

**Gene Cloning/Genetic Engineering:**

The methods of cell fusion and recombinant DNA technology have led to the expansion of industrial biotechnology [6]. The novel recombinant DNA technology methods are highly specific and well controlled and provide unlimited opportunities for the production of novel combination of genes. This method allows increasing the product yield by the amplification or modification of the specific metabolic steps or by removal of metabolic bottlenecks in the different pathways. The genetic engineering allows the manipulation of microorganism by the addition of novel properties to the industrial microorganisms. This is done to facilitate the production of novel and valuable compounds. The novel products include the synthesis and excretion of the enhanced range of enzymes, human growth hormone, insulin and interferon. The genetic engineering involves the manipulation of DNA by the isolation and recovery of the genes from the donar organism genome. This is a good alternative over traditional methods of strain improvement such as mutagenesis and genetic recombination. There has been considerable success in the overproduction of a variety of fungal proteins but limitation at the level of transcription is evident. Genetic engineering has been used extensively for cloning of amylase producing strains, mainly alpha amylase and glucoamylase.

Strain modification has been revolutionized by DNA transformation strategies but it remains difficult to clone unidentified genes. Thus, mutation and selection will persist as an integral part of many breeding programme.

**Mutagenesis**:

It is a conventional tool for strain improvement [6]. This is an effective method of improvement of industrial microorganisms and is done by subjecting for the repeated round of mutagenesis, selection and screening of suitable survivors. The mutation is considered to be a natural event as it can occur naturally or be induced. It occurs from a physical change in DNA by the change in the number of copies of an entire gene/chromosome [40]. This involves deletion, insertion, duplication inversion or

translocation of a piece of DNA. Mutagenesis has been used by several workers as a tool of protein engineering to achieve strains with higher enzyme productivity or desired characters [41]. Screening mutagenesis of enzyme overproducing strains is very important in improving the efficiency and economics of the industrial process [30]. Traditionally, strain development involves a laborious approach with regard to identification of superior isolates from a mutagen-treated population [42].

The mutagenesis induce modifications of the base sequence of DNA and cause base pair substitution, frame shift or deletions [6]. These also occur by the means of transposes delivered by suitable vectors. These methods are utilized in a limited manner because they can cause either loss of undesirable characteristics or by increasing the production. The most noticeable example of mutagenesis is the removal of the yellow colour of early penecillin preparations caused by chrysogenin, a yellow pigment produced by *Penicillium chrysogenum* [6]. The process of mutagenesis is also effective for increased yield of penecillin, aminoacids etc. The best example of mutagenesis is production of mutant of *Penicillium chrysogenum* in which the yield of penecillin is increased 550mg/l to 7000mg/l [41]. The process of mutation occurs spontaneously or after induction with mutagenic agents (mutagens). The use of different mutagenic agents for strain improvement was demonstrated by Parekh et al. [32].

**(a) Spontaneous mutation:** The rate of spontaneous mutation is slow but it can be increased by the use of mutagens [6]. The rate of spontaneous mutation depends on the growth conditions of the organism and the mutation frequency (proportion of mutants in the population) can be significantly increased by using mutagenic agents.

**(b) Induced mutations:** The mutagens are either physical or chemical in nature. **Chemical mutagenesis** involves the use of N-methyl-N'nitro-N nitroguanidine (NTG), nitrous acid, ethyl methyl sulphonate. Ethidium bromides intercalate the double stranded DNA and deform or affect the various biological processes. **Physical mutagenesis** involves the UV rays, gamma rays X-rays. Among the physical agents, UV treatment is widely used in industries as it is very effective and does not require any equipment. Agrawal et al, [43] showed UV to be a potent mutagen. The specific industrial production of enzymes is increased mainly due to UV treatment method. The UV radiation (Wave length 200-300 nm) with an optimum at 254 nm, which is the absorption maximum of DNA, is used. The most important products of UV action are dimmers (thymine-thymine, thymine-cytosine and cytosine-cytosine) formed between adjacent pyrimidines or between pyrimidines of complementary strands, which results in crosslink. UV mainly induces transitions, transversions, frame shift mutations and deletions. UV rays excite electrons in the molecule which leads to the formation of extra bonds between adjacent pyrimidine molecules. UV rays are known to be disparaging but they are capable of production of mutants with improved performance and ability for better adaptation to the environment.

Raju et al. [44] studied strain improvement of *Aspergillus niger* for glucoamylase by physical (UV) and chemical mutagens (Ethyl methyl sulphonate and ethidium bromide) and reported that the mutant strains of *Aspergillus niger* has a better ability for the glucoamylase production. Vu et al. [33] used repeated and sequential mutagenesis to mutate fungal strain having more RSDE activity. Vu et al. [45] studied the hyper production of raw starch digesting

enzyme by mutant *Aspergillus* sp. and found that the RSDE production was improved 2 folds higher than the wild type. The various mutagenic agents such as γ rays of Co60, UV and NTG were combined for the mutation of fungal strain and high level of RSDE. Irfan et al. [15] studied UV mutagenesis of *Aspergillus niger* for enzyme production in submerged fermentation. Pei-Cheng *et al*, [30] studied the UV and 60Co-γ-ray combined mutagenesis of *Aspergillus niger* for the enhancement of glucoamylase activity. Ghani et al. [2] reported enhanced production of glucoamylase in *Bacillus licheniformis* by mutation and it was found that the enzyme activity increased nearly two-fold in mutants as compared to the wild strains. Imran et al. [9] studied the effect of chemical mutagenesis on *Aspergillus niger* for the production of glucoamylase. The use of different mutagens for increased production of enzymes by various fungi is illustrated in Table 2.

**Table 2: Use of different mutagens for increased production of enzymes by various fungi**

| Name | Treatment | Enzyme activity | Reference |
|---|---|---|---|
| *Aspergillus oyzae* | Combined treatment of UV, Nitrous acid & EMS treatment | 2.1 fold increased α amylase activity | [47] |
| | Random mutagenesis | α amylase activity | [51] |
| *Aspergillus niger* | UV treatment | 2 fold increased CMCase activity and 3 fold increased Fpase activity | [15] |
| | UV treatment | 2.53 times increased lipase activity | [48] |
| | Combined tratment of UV, EMS (Ethyl methyl sulphonate) & Ethidium bromide | 2-4 fold increased glucoamylase activity | [44] |
| | Combined treatment of UV and $^{60}$Co-γ-ray | 13.7 fold increased glucoamylase activity | [30] |
| | Ttreatment of UV and EMS | 2.01 fold and 1.53 fold higher acid protease activities by UV and EMS respectively. | [52] |
| | UV treatment | 156% more lipase production | [53] |
| | γ- irradiation | 2-2.5 fold glucoamylase | [54] |
| *Penicillium chrysogenum* | UV treatment | High cellulose activity | [55] |
| *Rhizopus* sp. | UV treatment | High lipase activity | [56] |
| | UV treatment | Increased glucoamylase | [57] |
| *Aspergillus awamori* | Combined treatment of UV,N-methyl, N-nitro, N-nitrosoguanidine, dimethyl sulphate, EMS (Ethyl methyl sulphonate), Ethidium bromide and Nitrous acid | Increased amyloglucosidase | [58] |
| | Combined treatment | Increased amyloglucosidase | [59] |
| *Aspergillus* sp. | Combined treatment of γ rays of Co$^{60}$,UV treatment and NTG (N-methyl-N'- nitrosoguanidine) | 2 fold increased RSDE (raw starch digesting enzyme) activity | [45] |

| | | | |
|---|---|---|---|
| *Thermomyces lanuginosus* | UV and NTG treatment | 7 fold α amylase and 3 fold glucoamylase activity | [60] |

Singh et al. [46] used physical, chemical or combination of physical and chemical mutagens to isolate mutants of choice. Mishra et al. [34] studied that UV and nitrous acid treatment increased the production of Xylanase enzyme by *Acinetobacter* sp. Abdullah et al. [47] studied random mutagenesis for enhanced production of alpha amylase by *Aspergillus oryzae* HB-30. Sadana et al. [48] studied 2.53 times higher lipase activity of *Aspergillus niger* by nitrous acid induced mutation. Karanam *et al* [49] studied combined mutation studies using UV, $HNO_2$ and NTG on lipase activity from *Aspergillus japonicus* MTCC 1975 and found 127, 177 and 276 % higher lipase yield than parental strain respectively. Nadir et al. [50] also reported the combination of two mutagenic agents for reliable mutagenic rationale.

**(c) Site directed mutagenesis-** The overall mutability and mutation rate of specific genes are enhanced by the recent method of directed mutagenesis [40]. This is done to obtain the maximum frequency of the desired mutant types and requires the wide knowledge of the genes that control the target product and often a genetic map of the organism. Recently in vitro mutagenesis is used in combination with genetic engineering for the modification of isolated gene or parts of gene. This involves the change in the base sequence of DNA and changing the codon in the gene coding for that amino acid. It can be done by protein engineering method. The desired improvements might be increased thermo stability, altered substrate range, reduction in negative feedback inhibition, altered pH range. The method of site directed mutagenesis is illustrated in (Fig. 2).

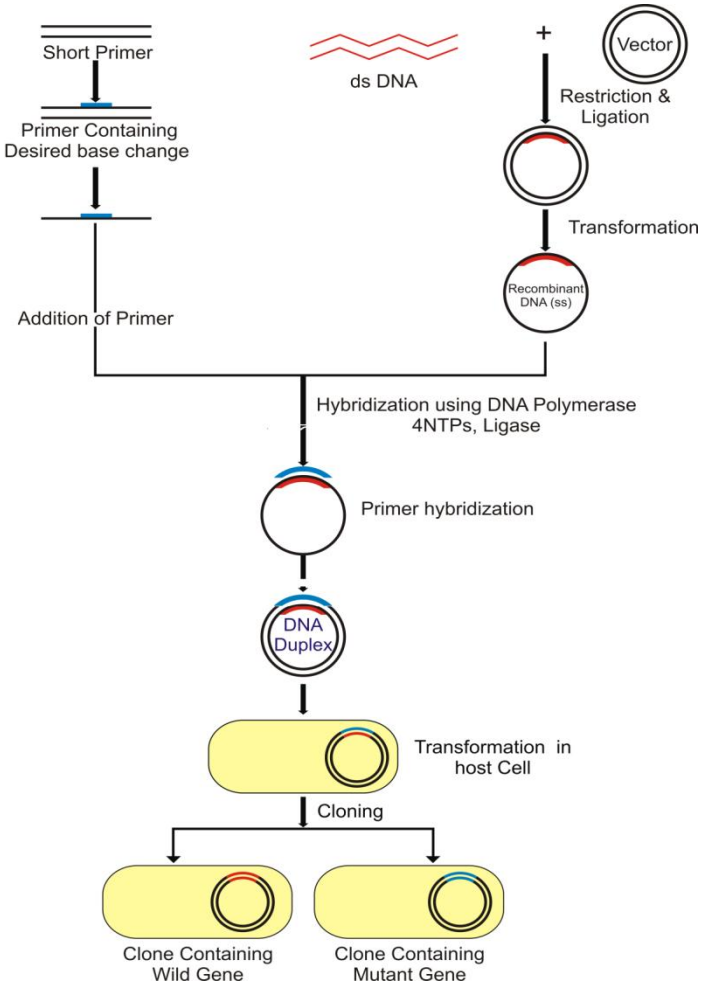


**Fig. 2: Site directed mutagenesis**

## CONCLUSION

With the growth of the biotechnology industry, fungi have been employed for commercial production of biocontrol agents as well as high-level production of enzymes, proteins and different metabolites. The strain improvement programs aims at the production of desirable strain having the ability of producing higher titres. Nowadays the discovery of new compounds and increasing the synthesis of these compounds is a challenging task. Therefore there is urge to investigate the use of novel methods for enhanced production of enzymes. The present paper aims to provide a review on glucoamylase from fungi, highlighting on recent advances on the mutational strategies for the improvement of industrial potential of fungal glucoamylase. Mutagenesis and recombinant DNA technology to some extent has resulted in tremendous increase in fermentation productivity and resulting decrease in cost. There is need to bridge the gap between basic knowledge and industrial application of the enzymes. However, future studies like the use of new genetic techniques like metabolic engineering and genome shuffling can be used for the increased production on large scale.

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