Mutation Effect of Chemical Mutagen Ethymthane sulfonate (EMS) on Some Local Yeasts

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Abstract

Thirty samples of orange juice were collected from local markets in Mosul / Iraq. Isolates were diagnosed after performing phenotypic, culture and biochemical tests. The results showed that the yeasts belong to the following species: *Rhodotorula rubra* 36%, *Trichosporon asahii* 16%, *Cryptococcus laurentii* 28%, and *Candida tropicalis* 20%. The susceptibility of isolates to six antibiotics Candizole (Cd), Clotrimazole (Ct), Fluconazole (Fc), Ketoconazole (Kc), Lamisil (Ls), and Nystatin (Nys) was studied. The results of the sensitivity test showed that *R. rubra* was resistant to all antibiotics used except for Lamisil (Ls). The rest of the yeasts varied among themselves in their resist antibiotics.

The chemical mutagen Ethyl Methanesulfate (EMS) at a concentration of 0.2 mg / ml on the vitality of the yeasts showed that the highest effect in the yeast *Crypto. laurentii*, with the killing severity reaching 4.47% while the lowest effected yeast was *Tricho. asahii*, with killing severity reaching 72%.

***Key words*:** Yeasts, Isolate, Orange juice, EMS.

1.Introduction

Yeasts are microscopic eukaryotic organisms located within the fungi kingdom. Their dimensions range from 3-10 µm in diameter to 5-30 µm in length [1]. It belongs to three fungal subdivisions: Ascomycota, Basidiomycota, and Deuteromycota, 1.500 species have been described today [2]. It spreads in many foods and can have a major or secondary role in the spoilage of some foods. The latter case results from the fact that it grows at a relatively low rate compared to bacteria [3]. It is considered one of the microorganisms that some of them can be found in extreme conditions, whether in terms of high glucose concentrations, as yeasts grow in them that like high permeability pressures Saccharophiles, including *Saccharomyces rouxii*, which causes spoilage of foodstuffs with high concentrations of sugars [4]. The low pH is 5.5 or less, and thus leads to significant economic losses as well as health problems, as it causes food poisoning and cancers due to the secretion of mycotoxins such as Aflatoxins [8]. Several studies and research have been conducted to investigate yeasts in juices. Obasiet *et al*. [5] observed the presence of *Candida kruesi*, *C. zeylanoides*, *C. parapsilosis*, *C. norvegensis*, *C. lusitaniae*, *C. parapsilosis*, *Rhodotorula minuta*, *Kodamea* sp. , *Geotrichum* sp. in natural and canned orange juice. Mathla *et al*. [6] was able to isolate the yeast *C. guilliermondi*, *C. lusitaniae*, *C. fumata*, *C. zeylanoides*, *C. krusei*, *Geot. capitatum* and *S. cerevisiae* from foodstuffs with high sugars content included fruits juice, apple jam, apricot jam, cherry jam, dates and others. While Chatterjee *et al*. [7] different types of yeasts in orange juice, apple juice, pineapple juice, grapes juice, mango juice and sugarcane juice.

Mutagenes work to bring about a change in the arrangement of nitrogenous bases, whether by deletion, addition, or substitution [8], Which is either substituting a base for another place from the same group and this is called a transition, such as replacing a purinian base with another place in the same group or a base Primidine is another place, but if a purine base is replaced by a premidine base or vice versa, the process is called transversion, which leads to a change in the composition of the gene and thus a change in the composition of the protein that encodes it, which may result in a change in characteristics such as the inability to synthesize some amino acids or vitamins or the emergence of allergy or resistance to some antibiotics and others [9]. Chemical mutagens are among the most important substances in causing genetic mutations. These mutagens include Nitrogen mustards, Nitrous Acid, Hydroxylamine, Hypoxanthine, Ethymthane sulfonate (EMS), Acridines, Nitrosoguanidine, 5-Bromouracil, and some other substances such as caffeine, Mitomycin, and others. EMS is one of the alkyating agents that attack the (CH3CH2) and methyl (CH3) group at the 7-site on the purine ring. This removes the base by displacing the DNA without affecting the sugar-phosphate column [10].

Due to the importance of this product to the consumer and the seriousness of its contamination with these microorganisms and the financial losses it causes, the current study focused on isolating and diagnosing some types of yeast that contaminate it. And studying the effect of chemical mutagens EMS on its vitality.

2.Materials and Methods

2.1Isolation of Yeasts

Colleted 30 samples of orange juice from local markets in Mosul city/ Iraq. From each sample, 1 gm was taken and a series of dilutions was performed from 10^{-1}-10^{-6}, after which 1 ml of the 10^{-5} and 10^{-6} dilutions were taken and implanted on Yeast Extract Malt Extract Medium (YM Agar) as it was spread on the surface using a glass diffuser (Spreader). With a volume of 3 plates for each sample, the plates were incubated at 28 C for 7 days until the emergence of yeast colonies.

2.2Diagnostic Tests

Morphological Characters of Colonies and Microscopic Examination

Isolates were grown on Malt Extract Agar (MEA) Medium and incubated at 28°C for 48 hours. Observations of phenotypic characteristics were recorded, and examined under a light microscope at 40X powers to observe the shape of the yeast cells.

Biochemical Tests

1. **Growth Test at 25 and 37 °C**

   The yeasts were grown on MEA medium by streaking method, and incubated at 25 and 37 ° C for 3-7 days. Record a negative result in the absence of growth or positive in the presence of growth.

2. **Test the usability of Nitrates as The Only Source of Nitrogen**

   The test was performed by cultivating the yeasts on the MEA solidified medium by Streaking method, incubated at 25 and 37 ˚C for 3-7 days.

3. **Determination of Ability to Resistance Glacial Acetic Acid**

   Inoculated Petri dishes containing solid medium Malt Acetic Acid (MAA) with part of pure culture of yeasts studied by Streaking method and incubated at 28 ˚C for 3-7 days. Infer the positive result with weak, medium, or dense growth and the negative result that no growth occurred.

4. Grow ability in low levels of water with higher levels of carbohydrates

Inoculated Petri dishes containing Malt Extract Yeast Extract 50% Glucose Agar (MY50G) medium with part of the culture of each isolate from the studied isolates by Streaking method and incubated at 28 °C. Observed the results after 3-7 days, the absence of growth indicates the negative result, while the occurrence of weak, medium or dense growth indicates the positive result.

5. Grow Ability in Low Levels of Water With Increased Level of Sodium Chloride

Isolates were inoculated by Streaking method on Malt Extract Yeast Gxtract 5%(or 10%) Salt 12% Glucose Agar (MY- 10-12) medium (MY10-12). And incubated at 28 ° C for 3-7 days. The result is positive when weak, medium, or dense growth occurs, and negative when no growth occurs at all [11].

6. The Ability of Yeasts to Form Mycelium Test

The test was conducted to find out ability of yeast to form the true mycelium and Pseudomycelium. Small flasks containing 20 ml of Sabouraud's Glucose Broth (SGB) medium were inoculated with a portion of pure culture yeasts. The flasks were incubated for 48 hours at 28 ° C. The yeasts were examined microscopically at force (40X) to note budding, cell morphology and mycelium type whether true or Pseudomycelium [12].

7. Diazonium Blue B (DBB) Color Test

Is one of the important tests to distinguish between Ascomycetes and Basidiomycetes yeasts. The isolated yeasts were grown on YM Agar food medium and incubated at 28 C for 10 days until the cysts were in the Ascomycetes. One to two drops of Diazonium Blue B reagent (DBB) reagent was added to the surface of the developing colonies and left for 2-3 minutes at laboratory temperature. The coloration of the colonies to a dark reddish-purple color indicates that they belong to the Basidiomycetes, which is (the positive result), but if the colonies were colored orange, this indicates that they belong to the Ascomycetes, which is the negative result [13].

2.3 Mutation Effect of Chemical Mutagen Ethyl Methanesulfate (EMS)

The method of Gjermansen [14] was used, 0.02 g of the EMS mutagen was dissolved in 100 ml sterile distilled water so that the mutagen concentration was 0.2 mg/ ml, 5 ml of which were transferred to a test tube containing 1 ml of the yeast cell suspension and the tubes were incubated in a vibrating water bath in Temperature 30 C for 70 minutes. Cells were deposited by central centrifugation using a Centrifuge for 15 minutes at 9000 rpm. Wash the precipitate with 10 ml of sodiumthia sulfate (2%) and centrifuge for 15 minutes at 9000 rpm. The washing process was repeated three times, then the sediment was suspended by adding 10 ml sterile distilled water, the tubes were shaken well and 0.2 ml of the suspension was spread by a sterile glass rod on the surface of the Petri dishes containing the solidified Sabourd's Glucose Agar Medium (SGA) medium at a rate of 5 dishes/ treatment with the presence of comparison dishes (control). The plates were incubated at a temperature of 28 C for a period of 7 days. The number of developing colonies in the five plates was calculated to be the total number of surviving colonies in 1 ml of mutated yeast suspension and the percentage killed according to the following equations:

$$\% \text{ Survivors} = \frac{The\ resulting\ number\ from\ \ mutagenesis\ treatment}{The\ resulting\ number\ of\ non-mutagenic\ dishes} \times 100$$

% kill = 100 - Percentage of surviving individuals

2.4 Antibiotic Resistance Test of Wild Type and Mutant Yeasts Isolates

For the purpose of determining the resistance of non-mutagenic and mutagenic yeast isolates using an EMS. The solidified Yeast Extract Peptone Glucose Agar (YPG) medium was prepared with the addition of antibiotics at the final concentrations mentioned in Candizole (Cd) ,Clotrimazole (Ct), Fluconazole (Fc), Ketoconazole (Kc), Lamisil (Ls), and Nystatin (Nys) at a concentration of 30 µg/ ml, the isolates were inoculated by Streaking method and the plates were incubated at a temperature of 28 °C for 48 hours .Isolates are antibiotic, as mutant isolates that are resistant to the antibiotics used here can be identified [15].

3.Results and Discussion

The current study demonstrated the presence of 25 isolates of yeasts out of a total of 30 orange juice samples belonging to four different genera. The most frequent yeast was *R. rubra*, which appeared in 9 isolates by 36%, and the least appeared was 4 isolates in *Trichosporon asahii* with 16%, Table (1). The reason for the high number of yeasts in juices can be explained by the speed of growth of yeasts, their possession of genes responsible for fermentation of sugars such as xylose, as well as their ability to grow in foods with a high level of glucose concentrations. These results are in agreement with other studies done on the juices [16], where they isolated strains of *S. cerevisae* from date juice and raisins, as well as Khattab *et al*. [17] who isolated the largest number of yeasts *Debaryomyces hansenii*, *S. cerevisiae*, *C. tropicalis*, and *Pichia kudriavzevii* from Orange juice, mandarin juice, mango juice, and sugarcane juice.

Table (1): Number of isolates and rates of infection with yeasts in orange juice samples

Yeast	The number of isolates	% Recurrence
R. rubra	9	36
Tricho. asahii	4	16
Cryptococcus laurentii	7	28
C. tropicalis	5	20
Total	25	100

3.1 Diagnostic Tests for Yeasts

Culture Characteristics and Microscopic Examination

The results of the diagnostic tests showed that the isolated yeasts belong to four different genera, which are as follows:

1. *R. rubra*: The color of their colonies was orange, 1.5 mm in diameter, round, mucous in texture, convex soft to the touch, shiny, with smooth edges, it appears under the microscope in the form of a spherical-lemon cell with a diameter of 12 µm with the presence of eosinophilic mycelium (1-A and B).

2. *Tricho. asahii* : Its colonies are white to pink, 3 mm in diameter, irregular, dry, diaphragmatic, cotton in texture, rough, opaque, flat, elevated center, with fringed edges, appear under the microscope a lemon-shape, with a diameter of 10 µm and are distinguished by their formation of true mycelium (1- C and D).

3. *Crypto. laurentii* : White colonies convex 1.2 mm in diameter, circular, leathery in texture, not mucous, shiny, with lobed edges and rough surface, under the microscope they appear oval to elongated in shape (7.0×2.0) µm with true mycelium (1-E and F).

4. *C. tropicalis* : White, frothy colonies 2.5 mm in diameter are round and convex, the edges of the colonies are smooth and shiny, under the microscope they look oval, their dimensions are (7.5×3.5) µm, Pseudomycelium formation (1-G and H).

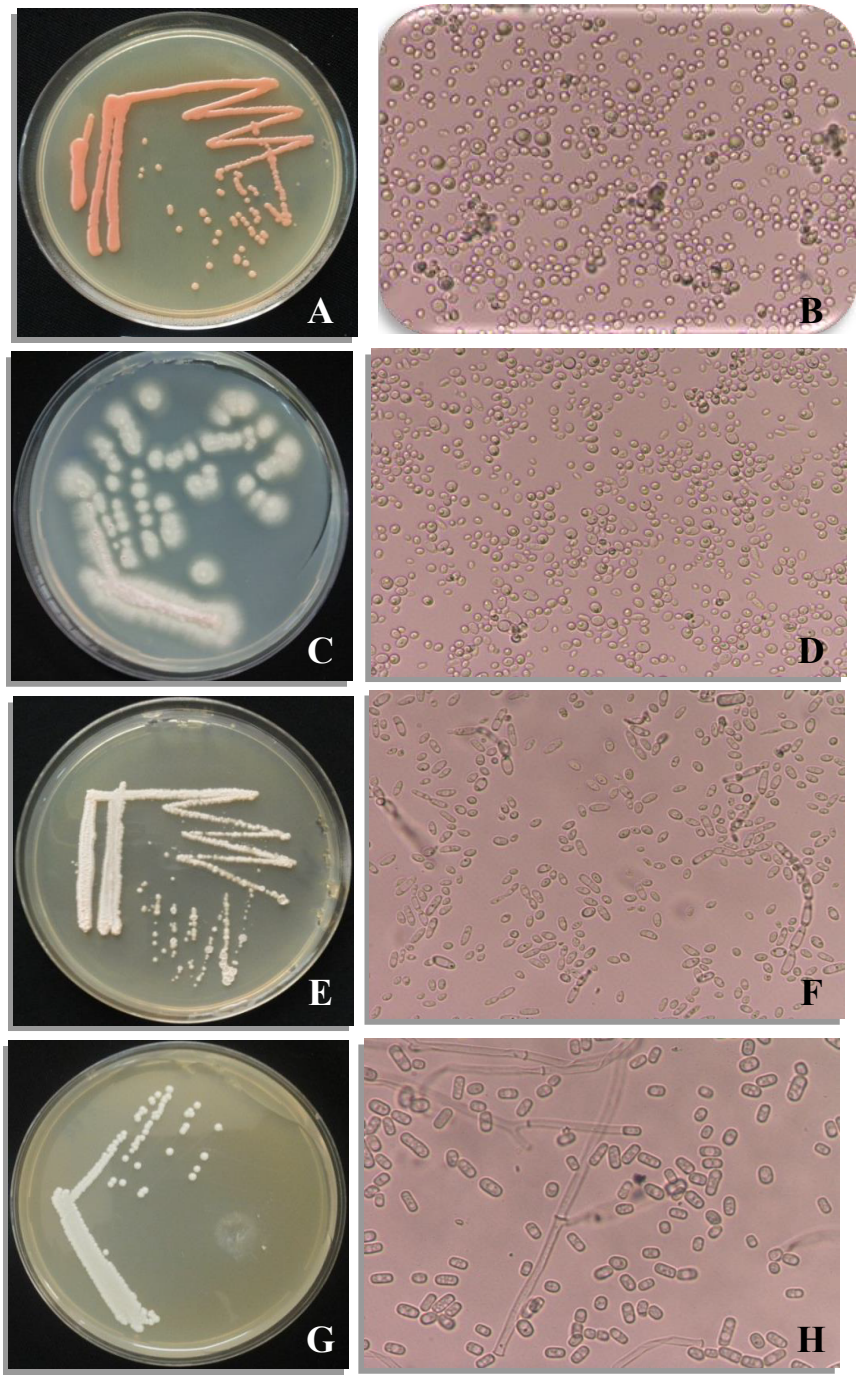

Figure (1): The Characteristics and Microscopic of yeasts

Where, A and B: *R. rubra*, C and D: *Tricho. asahii*, E and F: *Crypto. laurentii*, G and H: *C. tropicalis.*

Biochemical Tests

Growth Test at 25°C and 37 °C

The results of this test showed the ability of 3 isolates to 75% to grow at the tested temperatures with the exception of isolates *Crypto. laurentii* 25%, which showed a negative growth result at a score of 37˚C, Table (2), and this is consistent with what was reported by [18].

Test the usability of Nitrates as The Only Source of Nitrogen

The results came to demonstrate the ability of the two isolates *R. rubra* and *Crypto. laurentii*, 50%, weakly benefit from nitrate as the only source of nitrogen, as for the two isolates, *Tricho. asahii* and *C. tropicalis* (50%) was test negative in Table (2), and this result is consistent with what was indicated by [12].

Determination of Ability to Resistance Glacial Acetic Acid

It is noted from Table (2) that 3 isolates, 75%, were not able to resist snow acetic acid, except for *R. rubra* 25% as it grew thicker and this is consistent with the classification key mentioned by [12].

Grow ability in low levels of water with higher levels of carbohydrates

The results shown in Table (2) showed that 2 of the isolates (50%) were negative in this test, while the other two isolates (50%) were positive for the test and had weak growth.

Grow Ability in Low Levels of Water With Increased Level of Sodium Chloride

The current results revealed that *Crypto. laurentii* isolate 25% were test negative, while 5 isolates (75%) tested positive, and it ranged from *R. rubra* to weakly grown *Tricho. asahii* and *C. tropicalis* (Table 2). This is in agreement with [12].

Diazonium Blue B (DBB) Color Test

We notice in Table (2) that out of 4 isolates from the tested yeasts, only one isolate (25%) was positive in this test while 3 isolates were negative (75%). These traits are consistent with what Kurtzman and Fell [13] describe. It was mentioned that this test is widely used to distinguish between cystic and Yeasts.

Table (2): Biochemical Diagnostic Tests for Yeast Isolates

Diagnosed yeasts	Type of test						
	The ability to grow in						
	25˚C	37˚C	Presence of nitrates as a source (N)	Presence of glacial acetic acid	Low water level High for carbohydrates	Low and high water level for NaCl	DBB
R. rubra	+++	+	+	+++	+	+++	▪
Tricho. asahii	+++	+	-	-	+	+	▫
Crypto. laurentii	+++	-	+	-	-	-	▫
C. tropicalis	+++	+	-	-	-	+	▫

(-):There is no growth, (+): weak growth, (++):Medium growth, (+++):Dense good growth, (▪): Basidiomycetes, ( ): Ascomycetes

3.2 Mutation Effect of Chemical Mutagen Ethyl Methanesulfate (EMS) on The Vitality of Some Yeasts

The results showed mutagenesis of the four yeast isolates *R. rubra*, *Tricho. asahii, Crypto. laurentii*, and *C. tropicalis* using an EMS chemical mutagen at a concentration of 0.2 mg / ml had a clear effect on the viability of all susceptible yeasts, with the killing percentage being 61.72, 72, 47.4, and 60.5 % respectively (Table 3). It should be noted that chemical mutagens affect the nitrogenous bases that make up the basic unit for building the DNA (nucleotide), leading to permanent changes in the structural unit during its interaction with the nitrogenous bases. Its effect is to replace one nitrogen base with another, or alter the binding properties of the nitrogen base in such a way that it changes the binding forces with another base [19]. Fariss *et al*. [20] confirmed that the compound is one of the most effective substances in causing mutagenesis, as it does not act as a mutagen only, but rather is considered a toxic substance that kills yeast cells. And its effect on cell viability is directly proportional to its concentration in the medium [21]. Also, Anandarajah *et al*. [22] in their study that using a 0.28 M concentration of EMS increased the killing percentage to 50%. EMS was found to have a fatal effect on *S. cerevisiae* cells, and exposure to them for 45 minutes led to an increase in the killing rate to 43% [23]. Bessadok *et al*. [24] observed in their study that exposing the yeast cells *C. tenuis* CtTun15, *D. hansenii* DhTun 2015, *Tricho. Asahii* TaTun15, *Yarrowia lipolytica* YlTun15, and *R. mucilaginosa* RmTun15 for the EMS chemical mutagen for different time periods (15, 30, 45 and 60) min. The percentages of killed cells are directly proportional to the increase in exposure to all types of yeasts.

Table (3): The effect of the chemical agent EMS at a concentration of 0.2 mg / mL on the viability of the isolated yeasts

Yeasts	The Average of alive yeast		% Survivors	% Death
R. rubra	Control	32.4	38.27	61.72
	Treatment	12.4		
Tricho. asahii	Control	27.8	28	72
	Treatment	7.8		
Crypto. laurentii	Control	22.8	52.6	47.4
	Treatment	12		
C. tropicalis	Control	32.4	39.5	60.5
	Treatment	12.8		

3.3Resistance of Yeast isolates Studied to Antibiotics

It is evident from the observation of Table (4) that *R. rubra* was resistant to all antibiotics used except for Ls. The rest of the yeasts differed among themselves in their ability to resist antibiotics.Yeast resistance is due to its possession of a group of mechanisms that give the cell the characteristic of antibiotic resistance [25], including the inhibition of Ergosterol, which enters the synthesis of the cell membrane and the formation of holes that increase the loss of important substances to the outside of the cell [26]or the occurrence of a mutation in the genes encoding the enzymes that transport the drug to the cell as well as changes in the target protein that lead to an increase in its production and thus reduce the toxic effect of the drug [27]. This is in agreement with the Malla Obaida *et al*. [28]study that yeasts *R. minuta* BA78, *R. glutinis* BA83, *R. graminis*

BA1, *R. mucilaginosa* BA58, *R. mucilaginosa* BA75, *R. mucilaginosa* BA61, and *S. cervisiae* BA179 were resistant to Candizole, Fluconazole, Lamisil, and Nystatin and sensitive to Clotrimazole and Ketoconazole. The results of the susceptibility test for *C. colliculosas* showed resistance to all antibiotics used (Candizole, Clotrimazole, Fluconazole, Ketoconazole, Lamisil, and Nystatin) with the exception of the antibiotic Nystatin [29]. In another study conducted by Malla Obaida [30],*C. krusei* showed resistance to Candizole and Nystatin and sensitivity to the antibiotics Clotrimazole, Fluconazole, Ketoconazole, and Lamisil, while the yeast *C. utilis* showed resistance to all antibiotics except Nystatin, and the rest of the yeasts varied in their resistance and sensitivity to antibiotics.

Table (4): Sensitivity and Resistance Test of Yeast Isolates for Antibiotics

Yeasts	Antibiotics					
	Cd	**Ct**	**Fcz**	**Kc**	**Ls**	**Nys**
R. rubra	R	R	R	R	S	R
Tricho. asahii	S	R	R	S	R	S
Crypto. laurentii	R	S	S	R	S	R
C. tropicalis	R	S	R	S	R	S

R : Refer to the resistance status
S : Refer to the sensitivity status
Candizole (Cd) ,Clotrimazole (Ct), Fluconazole (Fc), Ketoconazole (Kc), Lamisil (Ls), and Nystatin (Nys).

3.4 Antibiotic Suceptibility Test of Mutated Yeasts Strains

The results of the study of resistance and sensitivity of yeast isolates mutagenic to the chemical mutagen EMS are shown in Table (5), as it is evident from the observation of the table that the yeast *C. tropicalis* has become resistant to all antibiotics. The yeast *Tricho. Asahii* showed sensitivity to all antibiotics except for Ct. Whereas, *R. rubra* and *Crypto. laurentii* became sensitive to all antibiotics used and retained the Cd antibiotic resistance status. Compared with non-mutagenic isolates, mutated isolates showed sensitivity or resistance to some antibiotics, and this may be due to a change in the genetic structure of parental isolates, which may have occurred in the genes carried on the plasmid or chromosome in the way that made these isolates sensitive or resistant to these antibiotics and here it is worth noting To the effect of mutagens used [31]. The EMS mutagen causes a single mutation of the type of mutations, double mutations of the type of transmission, transversion mutation, or transition mutations [32].

Table (5): Resistance and sensitivity of yeast mutant isolates using MNS for antibiotics

Yeasts	Antibiotics					
	Cd	**Ct**	**Fcz**	**Kc**	**Ls**	**Nys**
R. rubra	R	S	S	S	S	S
Tricho. asahii	S	R	S	S	S	S
Crypto. laurentii	R	S	S	S	S	S
C. tropicalis	R	R	R	R	R	R

R : Refer to the resistance status
S : Refer to the sensitivity status
Candizole (Cd) ,Clotrimazole (Ct), Fluconazole (Fc), Ketoconazole (Kc), Lamisil (Ls), and Nystatin (Nys).

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