ISOLATION AND CHARACTERIZATION OF THE YEASTS OBTAINED FROM NATURAL CAVES OF INDIA FOR BIOFUEL PRODUCTION

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Abstract: Fossil Fuels are one of the major contributors of ever increasing levels of Pollution index. Bioethanol can be promising replacement for this petroleum based fuels and can also possibly solve the issue of energy security in nearby future. One of the major hurdles of bioethanol industry is search for robust ethanol producing strains of microorganisms. Exploration of newer and less studied habitats like cave can prove to be important sources for such newer strains.
In the current study twenty four cultures were isolated from three natural caves of India. The cultures were studied for their cultural, morphological, microscopic and biochemical properties. The results were compared with that of reference strains *Saccharomyces cerevisiae* MTCC 170 and *Zymomonas mobilis* MTCC 2427 obtained from MTCC, Chandigarh. The isolates were screened for alcohol tolerance, osmotolerance and acetic acid tolerance. Most of the strains showed growth at 40-50% of the sugar concentration while three strains showed growth at 80% sugar concentration. In case of alcohol tolerance majority of the isolates were able to grow at 2% of alcohol whereas few of them showed the potential of growing at 5, 10 and 15 percent of alcohol. Isolates showing good results were studied for bioethanol production from various substrates. Fermentation was carried out with optimum conditions. Growth and fermentation kinetics were calculated for stationary fermentation. The results were compared with that of the above mentioned reference strains.

Keywords: Biofuel, Yeast, Cave Microbiology.

Introduction: Cave microbiology is the branch of geomicrobiology that deals with the study of microorganisms inhabiting cave environments. In the recent years the science of cave microbiology has mirrored the meteoric rise of microbiology as a science with a new insight suggesting that cave microorganisms may be involved in various important processes [1]. Very little amount of investigations have been done in the study dedicated to yeast ecology of caverns and their role in industrial and environmental processes [2].

Since ancient times, the only source of ethanol for domestic and industrial use is from the fermentation of sugars by yeast. The basic reaction involves the breakdown of glucose to carbon dioxide and ethanol [3].

Henry Ford once quoted that ethanol will be “the fuel of the future”. He also added that “The fuel of the future is going to come from various substrates like apples, weeds, sawdust and almost anything. There is fuel in every bit of vegetable matter that can be fermented” [4]. The significance of his futuristic vision is taking realistic shape in the form of fuel industry which is now looking towards waste to ethanol technology.

One of the major challenges of the ethanol industry is search for better strains of the organisms for continuous production with better yield. Osmotolerance, ethanol tolerance and ability of the yeasts to utilize sugar substances are some of the important properties for their use in industrial ethanol production [5][6].

The project aims at isolation of yeasts from three natural caves of India and screening them for the ethanol production from pure substrates, also their screening for cellulolytic activity.

Materials and Methods:

Sampling: Cave samples were collected from microbial communities covering a cave wall area. Samples were taken by either swabbing the suspected surface area of the rock or by scraping off colonies with a sterile scalpel. The samples were represented by a mixture of white, yellow, grey, greyish blue, black, brown and pink formations or macroscopic colonies on the cave surface [7]. After collection, the samples were stored in transport medium till its processing for isolation of the organisms.

Isolation of the Yeasts: The samples were streaked on Yeast Glucose Chloramphenicol agar (Himedia) plates for selective isolation of the yeast. Plates were further incubated at room temperature for 5-7 days and were observed until visible growth is seen. The pure colonies obtained were then maintained on Sabaraud's agar slant.

Standard Strains: The reference strains *Saccharomyces cerevisiae* MTCC 170 and *Zymomonas mobilis* MTCC 2427 were procured from MTCC, IMTECH, Chandigarh, India.

Screening of the Yeast for Ethanol Production: The isolates were inoculated in the Sabarauds broth and incubated for 48hrs. After incubation the tubes were centrifuged at 10000rpm for10 minutes. Alcohol production ability of the isolates were checked by qualitative detection of ethanol by Potassium dichromate method [8]. 1ml of the supernatant of centrifuged culture was made upto 5 ml volume with distilled water. Then 1 ml of Potassium dichromate reagent was added. All the test tubes were kept in ice bath and 4 mL of conc. Sulphuric acid added to each tube gently through the walls. Development of green colour due to presence of primary or secondary alcohol confirms the production of alcohol in the broth.

Determination of the Ethanol Tolerance of the Isolates: Ethanol tolerance of the isolates was determined based on visual assessment of turbidity and viability in a tube of basal medium containing 10g/L Bacteriological Peptone; 2g/L KH_2PO4; 50 g/L dextrose. 0.1ml of the yeast suspension with optical density of 0.1 was inoculated in 10ml of sterilized basal medium with varying concentrations of ethanol ranging from 1-20% at intervals of 1%. Tubes were incubated for 48 hours at room temperature. After incubation tubes were observed for turbidity or sedimentation which indicates growth and consequently ethanol tolerance [9] [10].

Determination of the Osmotolerance of the Isolates: 0.1ml of Yeast isolates were inoculated in 10ml of sterilized basal medium with varying concentrations of dextrose ranging from 5-80% at intervals of 5% and incubated for 48hrs at room temperature. After incubation tubes were observed for turbidity or sedimentation which indicates growth and consequently sugar tolerance.

Acetic Acid Tolerance [11]: Basal medium broth containing 10g/L bacteriological peptone; 2g/L KH_2PO4; 50 g/L dextrose was prepared containing 1% of acetic Acid. Blank media without acetic Acid was used as a control. Then 0.1 ml of each isolate was inoculated in the broth and incubated at room temperature for 48 hrs.

Quantitative Screening of the Yeasts for the Ethanol Production [8-10]: The isolates were inoculated in the Sabarauds broth and incubated for 48hrs. After incubation the tubes were centrifuged at 10000rpm for10 minutes, Alcohol production ability of the isolates were checked quantitatively by Potassium dichromate method. The amount of ethanol produced was determined colorimetrically and with the help of standard graph of different concentration of Ethanol.

Identification of Yeast [12-15]: DNA was isolated from the yeast isolate. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of ITS gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose Gel. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with ITS1 & ITS4 primer using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The ITS sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program

Qualitative Screening Of The Yeasts For The Ethanol Production From Various Substrates: The substrate chosen for the study were dextrose, sucrose, jaggery, steam pretreated sawdust and Avicel (Microcrystalline Cellulose). The isolates were inoculated in the 25ml of medium containing 10g/L bacteriological peptone; 2g/L KH_2PO4; 5g/L of yeast extract and 50 g/L of respective substrates and incubated for 48hrs. After incubation the tubes were centrifuged at 10000rpm for10 minutes, Alcohol production ability of the isolates were checked by qualitative detection of ethanol by potassium dichromate method [8].

Fermentation [16]: Fermentation was carried out at pilot scale glass fermenter (Capacity 7L) in 5L of the sterile fermentation medium with the composition of 10g/L bacteriological peptone; 2g/L KH_2PO4; 5g/L of yeast extract and 60g/L sucrose, at room temperature. 25ml of the inoculum (Optical density was adjusted to 0.1) was added. Fermentation was carried out for 50 hours and different aliquots (5hr, 10hr, 24hr, 30hr, 34hr, 48hr and 50hr) were removed and checked for pH, amount of reducing sugars (DNSA method) and alcohol (Potassium dichromate titrimetric assay). Temperature was also monitored at every interval. Distillation of the final product was carried out at 88ºC and percentage of ethanol was determined [17].

Results:

Sampling and Isolation: A total of 92 samples were collected from three different natural caves of India, details of the sampling are given in Table 1.1. 24 isolates of yeast were obtained from the samples collected, details are given in table 1.2.

Table 1.1: Sampling Details

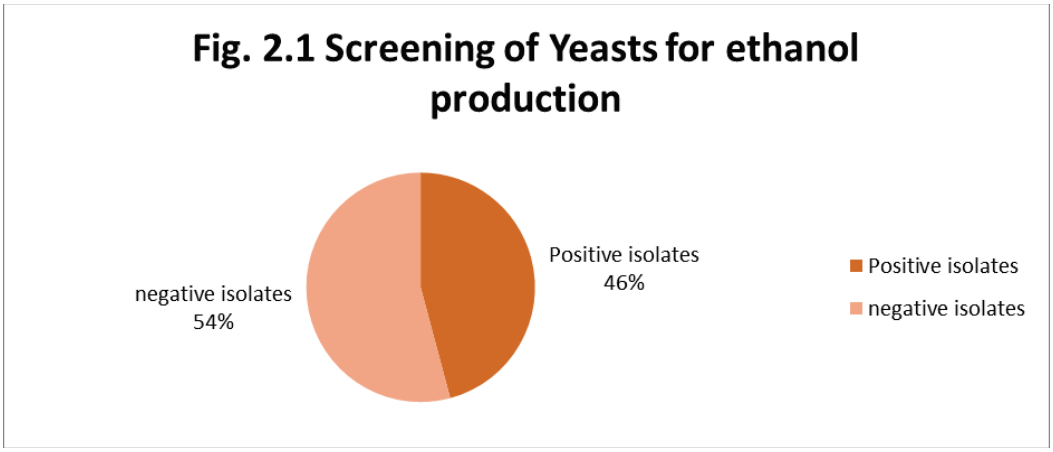
Cave Name	Location	Geographical Location	No. of surface Swabs
Panther's Cave	Matheran forest region, Neral, Dist. Raigad, Maharashtra	N 18.97° 59' X E 73.26° 18' Matheran, India	15
Naida Caves	Diu City, Union territory of Daman and Diu	N 20°42'38.65" X E 70°58' 51.24" Fudam, Diu, India.	52
Robber's Cave	Dehradun, Uttarakhand	N 31° 38′ 56″ X E 78° 2′ 0″ Malsi, Dehradun, India	20

Table 1.2: Isolation of the Yeasts

Sr. No.	Cave	Yeasts	Labeled as
1	Panther Cave	03	MCY1, MCY2, MCY3
2	Naida Caves	12	NCY1-NCY12
3	Robbers Cave	09	RCY1-RCY9

Screening of the Yeast for Ethanol Production: The ethanol production capacity of all 24 isolates in dextrose medium was screened. Only 11 isolates (MCY1, MCY2, MCY3, NCY2, NCY5, NCY9, NCY10,

NCY11, RCY1, RCY6 and RCY7) could carry out fermentation of the sugar to produce alcohol and consequently were selected for further experiments. Results are depicted in the Fig.2.1



Ethanol Tolerance of the Isolates: Detailed results of all the tolerance experiments are given in the table 3.1.

Table 3.1: Screening of Yeasts for Sugar Tolerance, Alcohol Tolerance and Acetic Acid

Isolate	Sugar Tolerance	Alcohol tolerance	1% Acetic Acid
MCY1	60%	15%	No Growth
MCY2	80%	10%	No Growth
MCY3	60%	10%	No Growth
NCY2	80%	20%	Growth
NCY5	10%	2.5%	No Growth
NCY9	80%	20%	Growth
NCY10	5%	2%	No Growth
NCY11	10%	2%	No Growth
RCY1	10%	2.5%	No Growth
RCY6	20%	2.5%	No Growth
RCY7	10%	2.5%	No Growth
RCY9	10%	2%	No Growth
Saccharomyces cerevisiae MTCC 170	80%	20%	Growth
Zymomonas mobilis MTCC 2427	20%	15%	No Growth

Yeast NCY2 and NCY9 were able to tolerate alcohol concentration of 20% (v/v). Most of the strains were found to be inhibited at alcohol concentration of 2 % and more. Results were compared with those of standard strains *Saccharomyces cerevisiae* MTCC 170 and *Zymomonas mobilis* MTCC 2427.

Osmotolerance of the Isolates: The yeast strains MCY2, NCY2 and NCY9 were able to grow at 80% (w/v) sugar concentration. MCY1 and MCY3 strains grew well upto 60% (w/v). Standard strain of *Saccharomyces cerevisiae* showed growth up to 80% of sugar concentration. Detailed results are depicted in the table 3.1.

Acetic Acid Tolerance: The yeast strains NCY2 and NCY9 were able to grow at 1% (v/v) Acetic acid. Standard strain of Saccharomyces cerevisiae also showed growth in 1% acetic acid, while growth of other isolates and Zymomonas was found to be inhibited. Detailed results are depicted in the table 3.1.

Quantitative Screening of the Yeasts for the Ethanol Production: In quantitative screening, amount of ethanol produced by isolate in 48 hrs was determined by dichromate assay. The data obtained was statistically evaluated and depicted in table 6.1.

Table 6.1: Quantitative Screening of the Yeasts for Ethanol Production With Statistical Evaluation

Isolate	**Mean** yield of ethanol in mg/ml	**Standard Error**
MCY1	**2.96**	**0.024**
MCY2	**2.93**	**0.012**
MCY3	**3.94**	**0.006**
NCY2	**4.94**	**0.019**
NCY5	**3.18**	**0.004**
NCY9	**5.05**	**0.024**
NCY10	**2.93**	**0.011**
NCY11	**2.94**	**0.007**
RCY1	**3.95**	**0.017**
RCY6	**1.98**	**0.006**
RCY7	**3.16**	**0.024**
RCY9	**3.03**	**0.012**
Saccharomyces cerevisiae MTCC 170	**5.04**	**0.007**
Zymomonas mobilis MTCC 2427	**3.94**	**0.019**

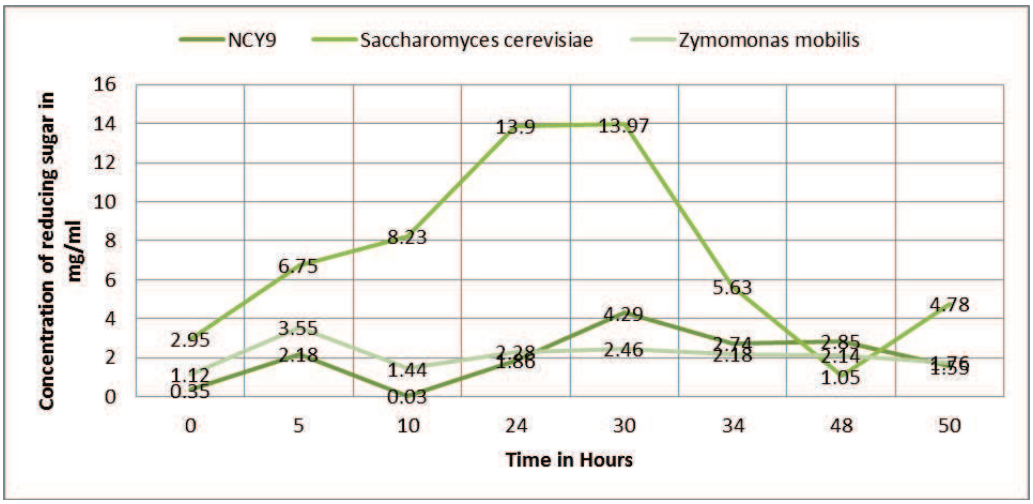
Qualitative Screening of the Yeasts for the Ethanol Production from Various Substrates: Yeast isolates were also checked for the ethanol production from various pure substrates as mentioned in materials and methods section. The results are depicted in table 6.1 wherein (+) indicates production of ethanol confirmed by Potassium dichromate method. Isolate NCY 9 was found to be most potent strain with substrate utility.

Table 7.1: Qualitative Screening of Ethanol Production from Various Substrate

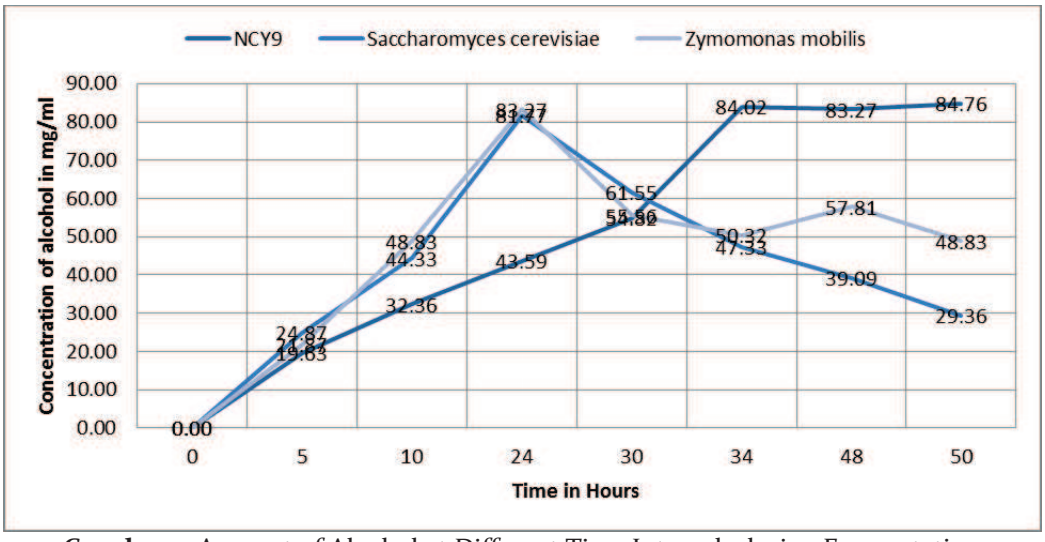
Isolate	Sucrose	Jaggery	Pretreated sawdust	Cellulose
MCY1	+	+	+	-
MCY2	+	+	+	-
MCY3	+	+	+	-
NCY2	+	+	+	-
NCY5	+	+	-	-
NCY9	+	+	+	-
NCY10	+	+	-	-
NCY11	-	-	-	-
RCY1	-	-	-	-
RCY6	-	-	-	-
RCY7	-	-	-	-
RCY9	+	+	+	-
Saccharomyces cerevisiae MTCC 170	+	+	-	-
Zymomonas mobilis MTCC 2427	+	+	-	-

Identification of Yeast: Based on nucleotide homology and Phylogenetic analysis the isolate NCY9 was found to be ***Candida tropicalis***

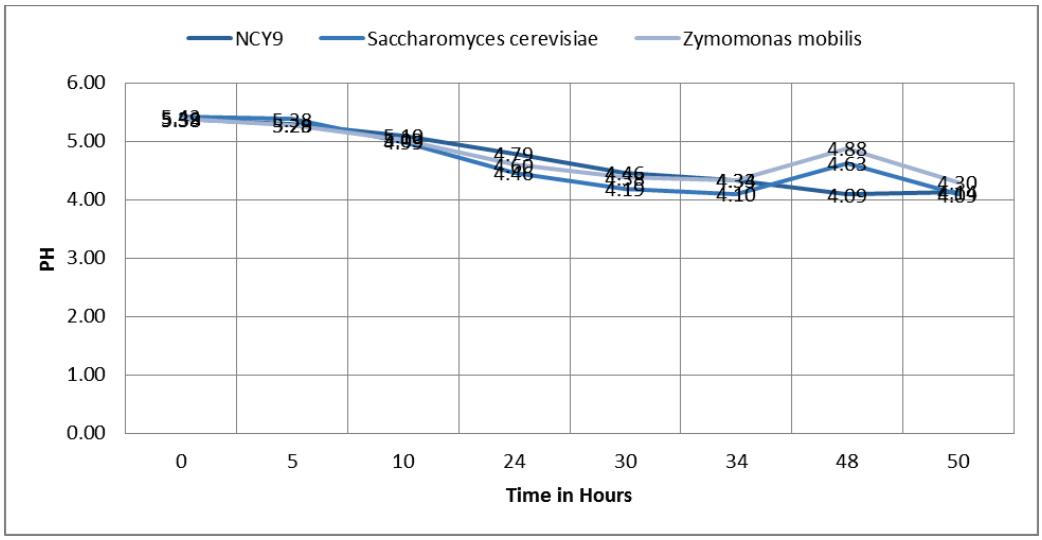
Fermentation: Fermentation was carried out at pilot scale glass fermenter (Capacity 7L) in 5L of sterile fermentation medium and the parameters checked at different time intervals. They are showm in Graphs 9.1-9.4.



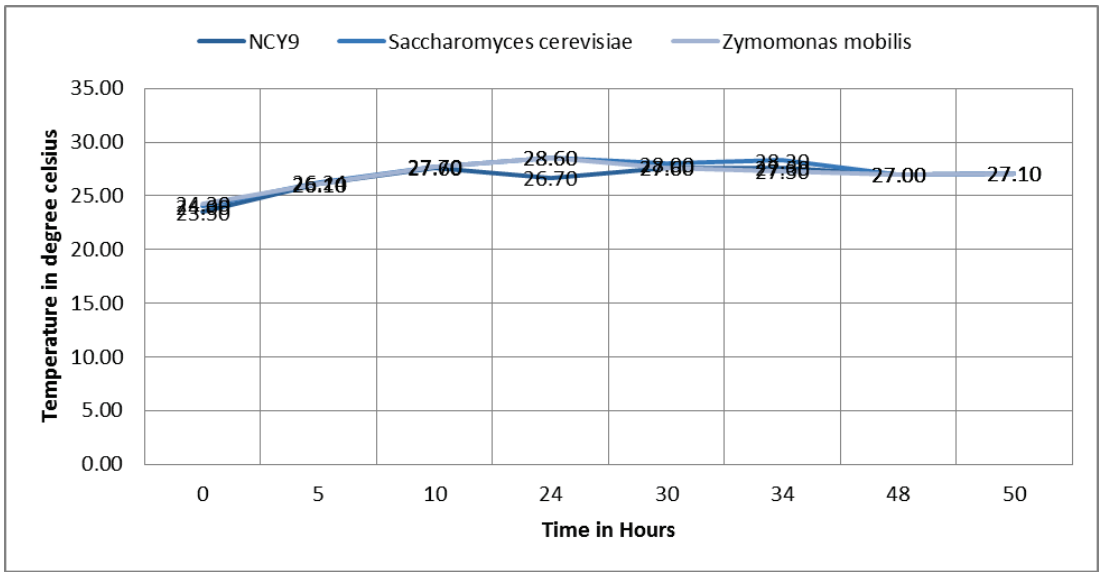
Graph 9.1: Amount of Reducing Sugar Released At Different Time Intervals During Fermentation



Graph 9.2: Amount of Alcohol at Different Time Intervals during Fermentation



Graph 9.3: pH of the Fermentation Broth at Different Time Intervals during Fermentation



Graph 9.4: Temperature of the Fermentation Broth at Different Time Intervals during Fermentation

The yield of ethanol was found to be better for *Candida tropicalis* (Isolate NCY9) with a yield of 84.02 mg/ml as compared to the yield of *Saccharomyces cerevisiae MTCC 170*
Zymomonas mobilis MTCC 2427 (please refer graph 8.3). pH of the medium throughout the fermentation was between the range of 5-4 with all three sets, temperature was close to room temperature which is also ideal for growth of yeast.

Conclusion: A total of 87 swab samples were collected from three different natural caves. Based on some of their morphological & physiological characterization, presumptive yeast isolates were selected. All the strains were tested for fermentation of carbohydrates for production of ethanol and out of 24 Yeast isolates obtained 12 were capable of fermenting sugars to ethanol. One of the most important aspects of ethanol fermentation is the capacity of yeast to tolerate ethanol concentration, because higher concentrations of ethanol inhibit alcoholic fermentation. The strains were screened for ethanol tolerance and NCY9 and MCY1 showed up to 15% ethanol tolerance.

Isolated yeast stains were able to tolerate up to 60-80% of sugar concentration. The yeast strains were also screened for their ethanol production ability from various substrates including pretreated sawdust, which could possibly be used as a substrate for biofuel production. The fermentation parameters like amount of reducing sugars utilized, amount of ethanol generated, pH etc were studied with the yeast strain NCY 9 and sucrose as a substrate. All the results were compared with the results of standard strains of the microorganisms. Isolated yeast was also found to better in productivity as compared to that of standard strain.

Molecular identification results revealed that the isolate NCY 9 is *Candida tropicalis.* Several studies also support use of *Candida tropicalis* over existing option of *Saccharomyces cerevisiae.*[18-20]
One of the major hurdles of the biofuel industry is search for better and novel strains of biofuel producing organisms. The project thus focused for new ecological niche i.e. natural cave for search of these isolates. The results obtained showed that yeast strain isolated can produce ethanol from variety of substrates and can also be employed for ethanol production from lignocellulosic substrate like sawdust.

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