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ORIGINAL PAPER

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Oxidative stress response of *Kluyveromyces marxianus* to hydrogen peroxide, paraquat and pressure

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Abstract The aim of this work was to study the oxidative stress response of *Kluyveromyces marxianus* to hydrogen peroxide (50 mM), paraquat (1 mM), an increase in air pressure (120 kPa, 600 kPa) and pure oxygen pressure (120–600 kPa) in a pressurized bioreactor. The effect of these oxidants on metabolism and on the induction of antioxidant enzymes was investigated. The exposure for 1 h of *K. marxianus* at exponential growth phase with either H_2O_2 or paraquat, under air pressure of 120 kPa or 600 kPa, induced an increase in both superoxide dismutase (SOD) and glutathione reductase (GR) content. SOD induction by the chemical oxidants was independent of the air pressure values used. A 2-fold increase in SOD activity was observed after 1 h of exposure to H_2O_2 and a 3-fold increase was obtained by the presence of paraquat, with both air pressures studied. In contrast, GR activity was raised 1.7-fold by the exposure to both chemicals with 120 kPa, but a 2.4-fold GR induction was obtained with 600 kPa. As opposed to *Saccharomyces cerevisiae*, catalase was not induced and was even lower than the normal basal levels. This antioxidant enzyme seemed to be inhibited under increasing oxygen partial pressure. The cells showed a significant increase in SOD and GR activity levels, 4.7-fold and 4.4-fold, when exposed for 24 h to 120 kPa pure oxygen pressure. This behaviour was even more patent with 400 kPa. However, whenever cells were previously exposed to low air pressures, low enzymatic activity levels were measured after subsequent exposure to pure oxygen pressure.

Introduction

Yeasts, as aerobic cells, have to face the toxic side effects of molecular oxygen, the production of reactive oxygen species (ROS). These species are generated during normal cellular metabolism (e.g. by the mitochondrial respiratory chain, or by H_2O_2-generating reactions catalysed by oxidases). ROS can also be originated from the presence of pro-oxidants, such as H_2O_2, paraquat or menadione in the medium, by exposure to ionizing radiations; or by an increase in the oxygen pressure (hyperoxia or re-oxygenation of hypoxic cells; Gille and Sigler 1995). These ROS, especially superoxide (O_2^-) and hydroxyl (OH) radicals, H_2O_2 and singlet oxygen ($^1O_2$), damage cellular components by oxidizing lipids, proteins and nucleic acids (Moradas-Ferreira et al. 1996). To protect cells against such reactive oxygen species, all aerobic cells have evolved enzymatic mechanisms to overcome these ROS. These include defence enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), several peroxidases and low molecular weight antioxidants (Galiazzo and Labbe-Bois 1993; Hohmann and Mager 1997). Under normal physiological conditions, antioxidant defence mechanisms are almost certainly adequate to maintain ROS at a basal level and to repair cellular damages (Moradas-Ferreira et al. 1996). The increased knowledge about stress protection and adaptation mechanisms in yeasts is of major importance. Due to the universal nature of the stress response, further insight into the response of yeasts is also relevant for improving the understanding of defence mechanisms in other cell types (Mager and Moradas-Ferreira 1993).

Compared with *Saccharomyces cerevisiae*, little is known about the oxidative stress response in *Kluyveromyces marxianus*. This is an important industrial yeast, both in classic applications (β-galactosidase production, biomass production from whey) and as host for heterologous protein production (Belem and Lee 1998; Kiers et al. 1998).

Cells pretreated with comparatively mild and sublethal stress situations induce adaptive responses and acquire tolerance to subsequent and more lethal stress. Such adaptive responses were observed in several bacterial cells (Lee et al. 1993; MacMichael 1988) and in eu-

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karyotic organisms, including yeasts (Izawa et al. 1995, 1998; Lee et al. 1995).

Knowledge about the effect of an increase in air pressure on the metabolism of different micro-organisms such as *K. marxianus* (Pinheiro et al. 2000), *Pseudomonas fluorescens* (Onken 1990), S. *cerevisiae* (Pinheiro et al. 1997), *Streptococcus lactis* (Taniguchi et al. 1992), *Thermus* sp. RQ-1 (Belo et al. 2000) and others (Onken and Liefke 1989) has significantly increased in recent years.

In order to learn more about the response of *K. marxianus* to oxidative stress, different ROS-generating agents were studied for their effect on cellular growth and antioxidant enzyme induction. The induction of antioxidant enzymes(SOD, CAT, GR) in response to H_2O_2, paraquat and air or oxygen pressure was investigated. The response of *K. marxianus* cells to pretreatment with air pressure and subsequent exposure to other oxidative stress agents was also investigated.

Materials and methods

Strain and maintenance

K. marxianus CBS 7894 was obtained from the Centaalbureau voor Schimmelcultures (Delft, The Netherlands). This strain was stored at –80 °C in *K. marxianus* medium with 20% (v/v) of glycerol. From these stock cultures, agar slants [2% (w/v)] were inoculated and maintained at 4 °C.

Mineral medium

The mineral medium consisted of: 5 g KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$ and 1 g yeast extract in 1 l of potassium phosphate buffer (0.2 M, pH 5.5). Lactose was used as the main carbon source at 10 g/l. After autoclaving (120 °C, 20 min), the medium was cooled to room temperature.

Operating conditions

Yeast cells were pregrown in 250-ml Erlenmeyer flasks filled to 10% of the experiment total volume of the mineral medium described above, containing 5 g lactose/l. Batch cultivations were carried out using a cylindrical stainless steel pressurized bioreactor with a total volume of 300 ml. The working volume was 150 ml and the agitation speed was 150 rpm. Air or pure oxygen was fed continuously into the bioreactor headspace at 1 vvm aeration rate. In all the experiments, the temperature was maintained at 30 °C.

H_2O_2 and paraquat treatment

Yeast cells were grown under pressure in the pressurized bioreactor for 15 h. At the exponential growth phase, H_2O_2 or paraquat was added to the culture, to a final concentration of 50 mM or 1 mM, respectively, and samples were taken after 1, 8 and 24 h of exposure to the chemical. Two different air pressures, 1.2 bar and 6 bar (120 kPa, 600 kPa, respectively), were used for each chemical concentration. Experiments without the chemicals were also performed with the same air pressures, as controls.

Pure oxygen pressure treatment

Yeast cells were grown in the pressurized bioreactor under different pure oxygen pressures. For the experiments using pretreatment with 1.2 bar air pressure, cells were grown for 15 h, after which pure oxygen at different pressures was introduced into the bioreactor continuously, until the end of the experiment. Samples were taken after 1 h, 8 h and 24 h of pure oxygen introduction.

Analytical methods

At appropriate intervals, culture samples were collected for analysis of cell dry weight and viability and for enzymatic assays (SOD, CAT, GR). Growth was measured by optical density at 620 nm and converted to grams of cell dry weight per volume (gCDW/l). Viability was determined with the methylene blue staining technique. Protein in cell extracts was measured using the Coomassie blue method (Bradford 1976). Bovine serum albumin was used as a standard.

Preparation of cell extracts

Cells were harvested from the cultures by centrifugation (5,000 *g*, 10 min), washed once with 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and resuspended in the same buffer, frozen and stored at –20 °C.

The cells were disrupted by shaking with 0.5-mm diameter glass beads for 15 min at 4 °C using a bead mill (Vibrogen V14; Edmund Bühler,). The beads and cell debris were removed by centrifugation at 5,000 *g* for 15 min at 4 °C. The supernatant was dialysed overnight in a 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA.

Enzymatic assays

Antioxidant enzymes assays were performed after dialysis of extracts. Total SOD was assayed by the method of McCord and Fridovich (1969), CAT was assayed using the method described by Beers and Sizer (1952) and GR was assayed according to Smith et al. (1988).

Results

Response to H_2O_2 and paraquat under air pressure

Cell growth

The effect of two different oxidative stress inductors, H_2O_2 and paraquat, on cell growth (Fig. 1) and viability (Table 1) was tested in the exponential growth phase. When cells were exposed to paraquat (1 mM) or H_2O_2 (50 mM), the cell growth was slowed down for both pressures used. A reduction in cell growth by the increase in pressure from 1.2 bar to 6.0 bar was observed both in the absence of the oxidants and in the presence of chemical oxidants. After the addition of the oxidants, the cells were able to resume growth; but they reached a higher final cell concentration at 1.2 bar than at 6.0 bar air pressure. Moreover, cells respond better to the paraquat than to the H_2O_2 exposure. It should be noticed that the chemical concentrations used were different, but for both reagents these are the highest values, compared with those tested by other authors on several micro-or-

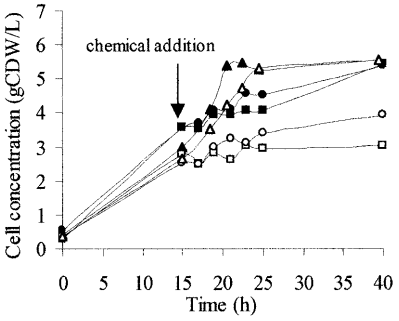

Fig. 1 Time course of cell concentrations for two different air pressure experiments, 1.2 bar (*black symbols*) and 6 bar (*white symbols*), when H_2O_2 (*squares*) or paraquat (*circles*) was added to the culture during the exponential growth phase. The controls (*triangles*) had no chemical addition. *gCDW/L* Grams cell dry weight/litre

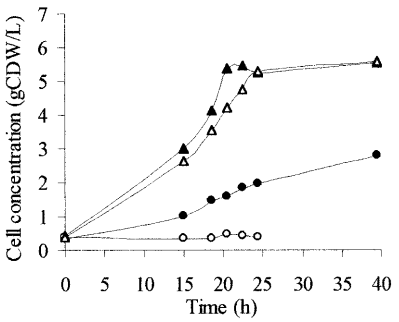

Fig. 2 Time course for cell concentrations for the different pressures studied: 1.2 bar (●) and 4 bar (○) of pure oxygen pressure, 6 bar (△) air pressure, control (▲; 1.2 bar air pressure)

Table 1 Percentage of cell viability with or without 24 h exposure to 1 mM paraquat or 50 mM hydrogen peroxide, under the same air pressure (1.2 bar or 6.0 bar). Each value is the average of three determinations from a representative experiment. Standard deviation did not exceed 5%

	Air pressure (bar)					
	H_2O_2		Paraquat		Control	
	1.2	6.0	1.2	6.0	1.2	6.0
Viability (%) after 24 h	75.0	78.9	73.0	68.6	95.2	93.2

Table 2 Percentage of cell viability after 24 h or 40 h of exposure to pure oxygen pressure or air pressure, with pretreatment (1.2 bar air) or without pre-treatment (1.2 bar or 4.0 bar pure oxygen pressure). *N.D.* Not determined

Viability (%)	O_2 Pure pressure (Bar)		1.2 Bar air pretreatment			
			O_2 Pure pressure (Bar)			Air pressure (Bar)
	1.2	4.0	2.0	4.0	6.0	10.0
After 24 h	73.6	25.0	91.2	54.4	52.6	92.8
After 40 h	53.2	N.D.	80.9	40.0	44.8	81.3

ganisms (Izawa et al. 1998; Kim et al. 1995; Lee et al. 1995; MacMichael 1988).

The cell viability started to decrease after chemical addition. However, at the end of the experiment, there were still 50% of viable cells (Table 1), which shows this *K. marxianus* strain is quite resistant to 1 mM paraquat and 50 mM H_2O_2.

Antioxidant defences

When exposing the cells of *K. marxianus* at exponential growth phase to either paraquat (1 mM) or H_2O_2 (50 mM) under the same air pressure (1.2 bar or 6 bar), the total SOD activity increased after 1 h of exposure to either chemical (Table 3). This activity was even higher when paraquat was used as the stress agent, for both air pressures. The total SOD activity increased 2-fold after adding the H_2O_2, for both pressures. In the case of paraquat, this antioxidant enzyme increased 3-fold, for both pressures. However, the SOD activity showed a slight decrease towards the end of the experiment, but the basal activity levels were never reached.

In the case of GR, an induction of 1.7-fold for 1.2 bar and 2.4-fold for 6 bar air pressure was observed after 1 h of exposure to each chemical. As concerns CAT, no significant changes in the enzyme activity were obtained, neither by the increased pressure, nor by the chemical oxidant addition.

Response to pure oxygen pressure

Cell growth

In order to investigate the effect of high oxygen pressure in cultures of *K. marxianus*, a set of experiments using increased pure oxygen were made and compared with the experiments with air. Respiration of oxygen, although essential to aerobic organisms for energy generation, also leads to the formation of harmful reactive oxygen species (Pahl and Baeuerle 1994). According to Onken and Liefke (1989), oxygen may have toxic effects on aerobic micro-organisms under oxygen partial pressures not much higher than 1.0 bar (in air).

When cells were grown under 1.2 bar or 6.0 bar air pressure, the maximum cell dry weight reached was very similar, 5.4 gCDW/l (Fig. 2). The viability did not decrease in either experiment (Table 2). However, in the experiment with 1.2 bar pure oxygen pressure, the same oxygen partial pressure in air with 6.0 bar produced a drastic decrease in growth, from 5.4 gCDW/l to 2.8 gCDW/l. As stated in Table 2, after 40 h of exposure to 1.2 bar pure oxygen pressure, there were only 53.2% of viable cells. With a higher pure oxygen pressure, 4.0 bar, after 24 h only 25% of the cells remained viable and the cells did not grow.

Table 3 Changes in the specific activities of defence enzymes, total superoxide dismutase (*SOD*), catalase (*CAT*) and glutathione reductase (*GR*), after 1 h, 8 h, or 24 h of exposure to 1 mM paraquat or 50 mM hydrogen peroxide under the same air pressure (1.2 bar, or 6 bar). Basal levels were considered as the values obtained in the control assay (1.2 bar air pressure without chemical). Standard deviation did not exceed 10%

Chemical	Air Pressure (Bar)	Time (h)	Activity (units/mg of protein)		
			SOD	CAT	GR
H_2O_2	1.2	1	136.7	0.077	0.77
		8	109.7	0.040	0.56
		24	60.0	0.060	0.32
	6.0	1	152.2	0.058	1.10
		8	135.9	0.025	1.02
		24	138.2	0.016	1.05
Paraquat	1.2	1	211.6	0.027	0.81
		8	144.8	0.044	0.44
		24	119.9	0.056	0.40
	6.0	1	230.1	0.120	1.13
		8	136.6	0.050	0.59
		24	N.D.	0.032	0.74
Control	1.2	1	71.9	0.066	0.48
		8	48.8	0.065	0.32
		24	36.6	0.064	0.29

Table 4 Changes in the specific activities of defence enzymes, after 1 h, 8 h and 24 h of exposure to pure oxygen pressure or air pressure, with (*1.2 Bar air +*) or without pretreatment

Gas	Pressure (Bar)	Time (h)	Activity (units/mg of protein)		
			SOD	CAT	GR
O_2	1.2	24	174.2	0.026	1.27
	4.0	24	226.2	0.059	1.41
Air	6.0	24	65.1	0.036	0.67
1.2 Bar air +	2.0 O_2	1	85.9	0.038	0.49
		8	82.8	0.048	0.50
		24	55.4	0.008	0.33
	4.0 O_2	1	70.0	0.087	0.43
		8	45.6	0.046	0.26
		24	59.5	0.023	0.35
	6.0 O_2	1	120.2	0.091	0.55
		8	109.5	0.066	0.48
		24	199.0	0.044	0.91
	10.0 air	1	104.0	N.D.	0.66
		8	135.2	0.098	0.86
		24	183.4	0.016	0.89
Air (control)	1.2	1	71.9	0.066	0.48
		8	48.8	0.065	0.32
		24	36.6	0.064	0.29

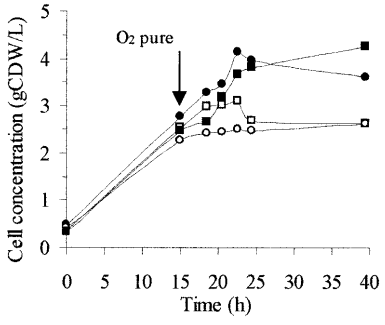

Fig. 3 Time course for cell concentrations for the different pressures studied. The cells were grown under 1.2 bar air pressure for 15 h. After this, a pure oxygen pressure [2 bar (●), 4 bar (□),6 bar (○)] or air pressure [10 bar (■)] was introduced into the bioreactor and maintained until the end of the experiment

Adaptive response to pretreatment

Cell growth

Cells were grown at low air pressure during the exponential growth phase, after which the pretreated cells were exposed to toxic levels of pure oxygen pressure (2 bar, 4 bar, 6 bar) or air pressure (10 bar) in the pressurized bioreactor. The 10 bar air pressure was used to be compared with the experiment with 2 bar pure oxygen pressure, since it has the same oxygen partial pressure as that in 10 bar air pressure. The results are shown in Figure 3.

For the pressures of 2 bar oxygen and 10 bar air, the cells had an adaptive response to the pretreatment, since the cells kept on growing after the exposure. At the end of the experiment, the cell concentration remained practically the same. This is not surprising since the oxygen partial pressure in both experiments was the same. This behaviour was not found for higher oxygen pressures, 4 bar and 6 bar, although in the case of 4 bar of oxygen, the cells had a short period of growth, 3 h. However, after this the growth stopped and the viability decreased. In contrast, in the case of 6 bar pure oxygen pressure, the cells could not cope with this pressure exposure and growth stopped. After 40 h, the fraction of viable cells was 44.8% (Table 2).

Antioxidant defences

In order to compare the effects of air and pure oxygen pressure on the induction of antioxidant defences of the strain *K. marxianus* CBS 7894, the SOD, CAT and GR activities were analysed. The results are illustrated in Table 4. Both total SOD and GR were induced at very high levels when pure oxygen pressure was used from the beginning of the cultivation time. After 24 h of exposure to the stress, GR was induced 4.4-fold and 4.9-fold for 1.2 bar and 4.0 bar, respectively. SOD had an increase of 4.8-fold and 6.2-fold for 1.2 bar and 4.0 bar. In contrast to SOD and GR, CAT activity slight decreased for the experiments made with pure oxygen pressure. In spite of the higher value of CAT activity obtained for 4 bar oxygen pressure, both results are lower than the activity values observed for the control. However, when cells were exposed to pure oxygen pressure after pretreatment, the total SOD and GR were much less induced than in the experi-

ments without pretreatment with 1.2 bar air pressure, allowing the cells to grow without being stressed. For the higher pressures investigated, 6 bar pure O_2 and 10 bar air, total SOD was induced 5-fold and GR 3-fold after 24 h of exposure to the stress agent. These high activity levels indicate that these highest values of pressure cause an excessive oxidative stress, leading to growth inhibition and cell viability loss. The low sensibility of CAT to oxygen pressure increase was once again demonstrated with this set of experiments. From the results presented, it is possible to infer that the pretreatment with 1.2 bar air induced a large increase in tolerance to toxic oxygen pressures.

Discussion

In this work, we describe the response of aerobic *K. marxianus* cells to oxidative stress induced by 50 mM H_2O_2, 1 mM paraquat and increased pressure.

Under air pressure, after a 24 h exposure to the chemicals, the cells were able to tolerate the concentrations imposed. However, the increase in air pressure to 6.0 bar followed by the chemical oxidants addition presented a strong stress condition for cell growth. In fact, the lowest final cell concentration was obtained at 6.0 bar and 50 mM H_2O_2 exposure. The results found for the cellular viability suggest that this *Kluyveromyces* strain is very resistant to both chemicals, for the concentrations used, which are considered to be lethal for other micro-organisms. For instance, the cells of *Streptomyces coelicolor* (Lee et al. 1993) are very sensitive to peroxide because, after treatment with 20 mM H_2O_2 for 30 min, only 0.1% of cells were viable. Also, in the work of Lee et al. (1995) at 40 mM H_2O_2, less than 10% of cells of *Schizosaccharomyces pombe* survived after a 1 h treatment.

High activity levels of SOD and GR were induced after 1 h of exposure to H_2O_2 or paraquat. These observations show that both antioxidant enzymes have an important role in the defence against the ROS originated. Agius et al. (1998) also reported that cells of *Saccharomyces cerevisiae* showed an increase in total SOD activity when grown in a medium containing 1 mM paraquat. In contrast, CAT seems to suffer a slight inhibition by H_2O_2 and paraquat for the concentrations studied. The high induction of SOD and GR might have balanced the CAT inhibition for this yeast strain, in defending the yeast cells against the stress which had been imposed. Izawa et al. (1998) suggested that glutathione recycling via GR reaction must be one of the mechanisms of the adaptive response to H_2O_2 stress. It should be noticed that the study of cell adaptation with different concentrations of chemical oxidants was not the aim of this work, since other authors have studied this subject. When both chemicals were compared, for 6 bar, it was possible to observe that the activity of GR, in the case of H_2O_2, was higher than in the case of paraquat. This was also observed in the work of Izawa et al. (1995) for a concentration of 0.2 mM H_2O_2 with *S. cerevisiae*. According to those authors, the intracellular GR plays an important role in the response of this yeast to H_2O_2, because this enzyme has a higher affinity with H_2O_2 than CAT. The results found in our work are consistent with the data obtained by Izawa et al. (1995).

It is known that pretreatment of cells with sublethal concentrations of toxic chemicals, such as H_2O_2, give the cells resistance against subsequent treatment with lethal concentrations of the same or another oxidative stress (Agius et al. 1998; Izawa et al. 1995, 1998; Steels et al. 1994). When pretreated with low air pressure, cells were able to grow and tolerate the stress imposed. When hyperbaric oxygen was used, the cells could not induce adaptation to a pressure higher than 4 bar. Previous studies on *S. cerevisiae* by Pinheiro et al. (1997) showed that growth was inhibited with the increase in pure oxygen pressure and was completely inhibited with 8 bar. A preliminary exposure to 1.2 bar pure oxygen might induce in cells the necessary adaptation to keep their growth (Izawa et al. 1995, 1998; Steels et al. 1994).

The results obtained in our work suggest that pretreatment with 1.2 bar air pressure induced in cells the capacity to tolerate high oxygen concentrations, for example, in the experiments with the same oxygen partial pressure (2 bar pure oxygen pressure, or 10 bar air pressure), the growth behaviour was very similar. In contrast, in the experiments without pretreatment (1.2 bar pure oxygen pressure, or 6 bar air pressure), there was a drastic difference in growth behaviour. This can be explained by the high oxygen transfer rate found for the experiment with pure oxygen pressure (Pinheiro et al. 1997). As a consequence of higher oxygen concentration, the cells may retard or stop their growth, depending on their sensitivity to oxygen (Onken and Liefke 1989).

The high activity levels found for SOD and GR in the experiments without pretreatment, with 1.2 bar and 4.0 bar pure oxygen pressure, suggested that the cells were strongly stressed. Taniguchi et al. (1992) found that when cells of *Streptococcus lactis* were exposed to 6.0 bar pure oxygen pressure, the SOD activity was more than twice as high as that under anaerobic conditions. Also, Westerbeek-Marres et al. (1988) found that cells of *Saccharomyces cerevisiae* induced high activity levels of SOD when grown in hyperoxic conditions. From the results presented, it is possible to conclude that the pretreated cells (in the exponential growth phase) were more resistant to oxygen pure pressure than the cells that were not pre-treated. Steels et al. (1994) suggested that the tolerance to oxidative stress, after pretreatment, was related to growth phase: in the stationary phase, cells were intrinsically more resistant to oxidative stress than cells in the exponential growth phase. The effect of increased air and oxygen pressures at different cellular growth phases will be studied in a future work.

Comparisons between the different oxidative stress inductors can be established. Both antioxidant enzymes, SOD and GR, were highly induced when the three different oxidative stress agents were imposed on the cells of *K. marxianus*. However, the activity of GR seems to respond better when oxygen is present in a high concentra-

tion than when another stress is used. A slight inhibition of CAT activity could be observed when oxygen was used. Kim et al. (1995) reported that the small amount of free radicals generated by paraquat in SOD-deficient cells of *S. cerevisiae*, could be sufficiently overcome by glutathione peroxidase but not by CAT.

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