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Novel chitinolytic potential of *Achromobacter denitrificans* isolated from fishery waste

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Abstract: *The Chitinase producing bacterial species identified as Achromobacter denitrificans was isolated from fishery solid waste collected from local fish market, Nashik, Maharashtra, India. The literature survey has not reflected the chitinase activity by Achromobacter denitrificans therefore this is reported for the first time. Growth in mineral salt medium with chitin as sole carbon source in shaking incubator at 30^0C lead to the production of enzyme exo- chitinase. The purified chitinase activity was found to be maximum at 35^0C and at pH 7.0. Added with Na^+, Cu^{++} and Ca^{++} chitinase activity increased but it was absolutely inhibited by Hg^{++}. Enzyme activity gradually increased as substrate concentration increased upto 7mM, further increase in substrate concentration did not influence the enzyme activity. This present isolate showed degradation of chitin under ambient environmental conditions and hence making it a potent candidate for industrial scale use and sustainable development.*

Key words*: Chitinase, colloidal chitin , metal ions, Sustainable development.*

I. Introduction

Chitin is the second most abundant natural biopolymer (De-hui Dai et.al, 2011), a polysaccharide composed of β-1, 4-linked N-acetyl- β-D-glucosamine units (Ahmadi et. Al, 2008; Shahidi et. Al, 2005). Which are composed of parallel- β, antiparallel-α and mixed-γ strands. (Svitil et.al, 1996). It is widely distributed in the coatings of many species, being a part of the cell wall of most fungi, the microfilaria sheath of parasitic nematodes, the exoskeleton of all types of arthropods and the gut lining of many insects (Ahmadi KA et.al, 2008). Chitin is always found crosslinked to other structural components, such as proteins and glucans (Gooday et. al, 1990). So far, it has been found that it has very little large-scale industrial use because of its extreme insolubility; cannot be absorbed or digested directly in the gastrointestinal tract (De-hui Dai et.al 2011). The annual global yield of chitin is assumed to be 1 to 100 billion metric tons, making chitin the second most abundant polysaccharide on the earth that can be obtained as a cheap renewable biopolymer from marine sources. It is biocompatible, biodegradable and bio-absorbable, with antibacterial and wound-healing abilities and low immunogenicity; therefore there have been many reports on its biomedical applications. Chitinases belong to glycosylhydrolase families 18 and 19 according to the classification made by Henrissat and Bairoch on the base of their amino acids sequences. These enzymes are present in a wide range of organisms such as bacteria, fungi, insects, plants and animals (Gomes et.al, 2001). The chitinases of the above-mentioned organisms play important physiological and ecological roles. Screening and isolation of organisms capable of producing chitinase is usually done on a medium containing chitin. Chitinases are enzymes that catalyse the hydrolysis of β-1,4-linked N-acetyl- β –D-glucosamine linkages present in chitin. Chitinases consist of endochitinase, exochitinase, β-N-acetylglucosaminidases and chitobiases (Gokul et.al., 2000). As chitin is a major component of fungal cell walls and is absent in plants, chitinases play a role in plant defence against pathogens. Supportive evidence for the defensive role of chitinases includes chitinase inhibition of fungal growth in vitro enhanced resistence to pathogens in plants that constitutively express high levels of chitinase and visualization of in vitro chitin breakdown(Gomes et.al, 2001). Chitin agar plate is used for isolating chitinolytic (Felse et.al, 2000) microorganisms and the plates were observed for a clearing zone surrounding the colony of microorganism.

Aim- The present study aims to investigate chtinase producing bacteria from fishery waste. The screening of isolates obtained for their enzymatic activity and the effect of temperature, pH, substrate concentration and metal ion concentration on enzymatic activity.

II. Materials And Methods

2.1 Isolation of chitinase producing bacteria by enrichment

Chitinase producing bacteria was isolated by enrichment of fishery solid waste. The enriched broth was serially diluted and spread on chitin agar plate containing (g/lit): chitin (8.0), KH_2PO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.5), NaCl (0.3), $CaCl_2 \cdot 2H_2O$ (0.05), NH_4Cl (0.5), peptone (5.0), yeast extract (1.0), $FeSO_4 \cdot 7H_2O$ (0.05) and agar (20.0), adjusted to pH 7.0 (Bansode et.al 2006). The plates were incubated at 30ºC temperature for 48 hrs. The isolated colonies obtained through spread plate method were sub-cultured on chitin agar slants, in duplicates, incubated and then stored at 4^{o}C (Annamalai et.al. 2010).

2.2 Preparation Of Colloidal Chitin

Colloidal chitin was prepared and modified according to the method of Lee et al. 2007 : 1 g chitin powder was added slowly to 20 ml of concentrated HCl and left at 4°C overnight with vigorous stirring (most probably in shaking incubator). The mixture was then added to 200 ml of 95% ice-cold ethanol with rapid stirring and kept overnight at 4°C. The precipitate was collected by centrifugation at 5,000 rpm for 20 min at 4°C and then washed with sterile distilled water until the colloidal chitin became neutral (pH-7.0).

2.3 Screening

The pure colonies maintained on chitin agar slants were streaked on colloidal chitin agar medium plates (1%) and then screened based on their ability to hydrolyze and grow on chitin plates due to production of chitinase. The plates were kept for incubation at 30°C for 48 hrs. The zone of clearance was measured and the culture giving significant result was selected for further analysis (Annamalai et. al. 2010 and Gohel et.al 2006).

2.4 Enzyme production

Chitinase production was achieved by culturing the organism into production medium containing (g/L): colloidal chitin 8.0, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.4, NaCl 0.5, $CaCl_2.2H_2O$ 0.05, NH_4Cl 0.5, peptone 5.0, yeast extract 1.0, and $FeCl_2.4H_2O$ 0.005, solution adjusted to pH 7.0. Culture medium was seeded with 5% inoculum and incubated at 30^{o}C for 96 hrs under aerobic conditions (a continuous shaking condition at 120 rpm/min) (De-hui Dai et. al. 2011 and Annamalai et. al. 2010). The culture broth from the shake flask was harvested after 72 hrs and the cells were removed by centrifugation at 10,000 rpm for 10 min. The supernatant was brought to 80% saturation with solid ammonium sulfate and left standing overnight at 4°C. The precipitate was collected by centrifugation at 12,000 rpm (4°C) for 20 min and re-dissolved in 50 mM pH 4.2 sodium-acetate buffer. The solution was dialyzed for the removal of ammonium sulfate in the same buffer for 12 h at 4°C (De-hui Dai et. al. 2011). The dialyzed partially purified enzyme was stored at 4°C till further analysis.

2.5 Enzyme assay

Chitinase activity was measured with colloidal chitin as substrate. Enzyme solution (1.0 ml) was added to 1.0 ml of substrate solution,which cointained 1.0% collidal chitin in 50mM posphate buffer (pH 8.0). The mixture was incubated at 30^{0}C for 20 mins. After incubation the mixture was subjected to centrifugation immediately at 10,000 rpm for 10 mins. The supernatant was used for analysis of reducing sugar using the dinitrosalicylic acid (DNS) method (Miller, 1959) with N-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of enzyme that produced 1µmol of reducing sugar per min. (Bansode et. al. 2006, De-hui Dai et. al 2011 and Annamalai et. al. 2010).

2.6 Effect of temperature on enzyme activity

In order to determine the optimum temperature, chitinase activity was examined at temperature range between 30^{0}C to 60 ^{o}C (Bansode et.al 2006), in 50mM phosphate buffer (pH 7.0).The activity of the enzyme was evaluated by incubating enzyme solution (1.0 ml) and 1.0% colloidal chitin (1 ml) at different temperatures for 1 hr and enzyme activity was assayed (Annamalai et. al 2010).

2.7 Effect of pH on enzyme activity

The activity of the purified chitinase was also measured at different pH values. The pH was adjusted using the following three different buffer system (50 mM): Acetate buffer (pH – 3.0 to 5.0), Phosphate buffer (pH – 6.0 to 8.0) and Carbonate buffer (pH – 9.0 to 11.0). The activity of the enzyme was evaluated by incubating enzyme solution (1.0 ml) and 1.0% colloidal chitin (1 ml) at different pH for 1 hr at 35^{0}C and enzyme activity was assayed (Bansode et.al 2006, Ahmadi et. al. 2008 and De-hui Dai et. al 2011).

2.8 Effect of metal ions on chitinase activity

Effect of different metal cations on chitinase activity were measured by the addition of the corresponding ion to the reaction mixture and the assay was performed under standard conditions. The tested

cations included the following corresponding salts: NaCl (Na+), $ZnCl_2$ (Zn^{2+}), $CuSO_4$ (Cu^{2+}), HgSO4 (Hg^{2+}) and $CaSO_4$ (Ca^{2+}). The effect of metal ions on the enzyme activity was determined by incubating 1 ml enzyme solution and 1 ml of 1.0% colloidal chitin with different metal ion concentrations (0.0 to 1.0 mM) at 30°C for 2 hr (Bansode et.al 2006 and Annamalai et. al 2010).

2.9 Effect of substrate concentration on chitinase activity

Different concentrations of colloidal chitin (0 mg/ml to 10 mg/ml) were incubated with 1 ml of enzyme solution at 30°C for 1 hr. Whole reaction mixture were suspended in 50 mM phosphate buffer pH 7.0. (Bansode et.al 2006 and De-hui Dai et.al. 2011).

III. Results And Discussion

1.1 Isolation and Screening

It was found that out of five bacterial isolates two S1 and S4 were promising regarding chitin degradation. Identification and confirmation of most efficient bacterial isolate S4 was achieved by Vitek-2 System Version: 05:02. Strain S4 was identified as *Achromobacter denitrificans* (Figure 1 and Table 1). The literature so far studied has no reports for any chitinolytic activity by *Achromobacter denitrificans*, therefore it is for the first time reported to degrade chitin.

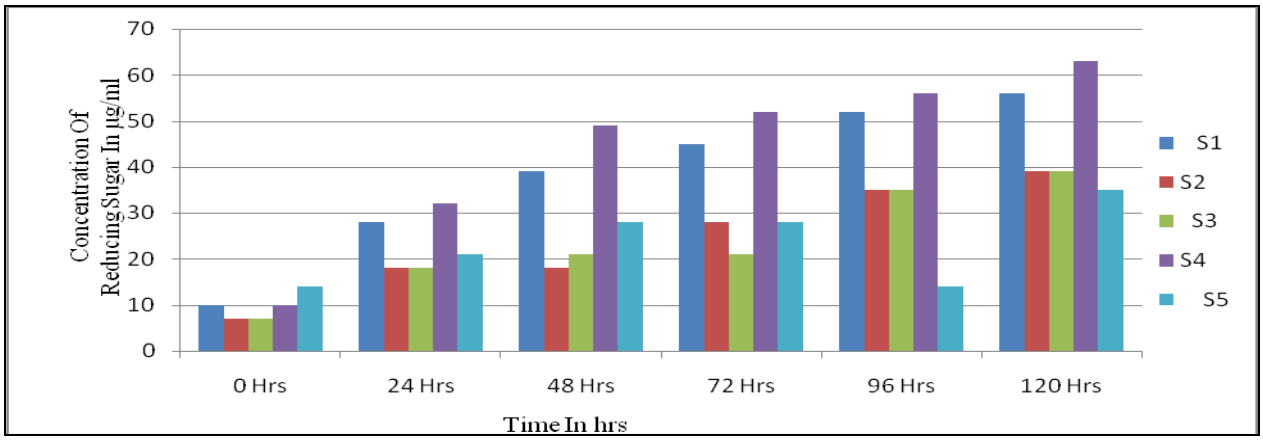

Figure 1: Screening of chitin degrading bacteria.

Table 1. Vitek-2 system report for identification of the organism.

Bac-test laboratory

Printed Oct 16, 2013 19:54 IST

bioMerieux Customer Laboratory Report Printed by Bactest

System # Bench WATER

Bionumber: 4042001101500051

Selected organism: Achromobacter denitrificans

Identification information	Card: GN	Lot Number: 241208540	Expires: Oct. 29, 2015 12.00 IST
	Completed: Oct 15, 2013 23:34 IST	Status: Final	Analysis Time: 10.25 hours
Selected Organism	88% Probability *Achromobacter denitrificans* Bionumber: 4042001101500051		Confidence: Intermediate

Biochemical details:

2	APPA	-	3	ADO	-	4	PyrA	+	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	-	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	-	79	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	+	64	ILATa	-			

Installed VITEK 2 System Version: 05:02 Therapeutic Interpretation guideline:

MIC Interpretation guideline: AES Parameter Last Modified:

3.2 Effect of temperature on chitinase activity and stability

The effect of temperature on chitinase activity obtained from *Achromobacter denitrificans* is shown in figure 2. In order to determine the optimum temperature of the chitinase enzyme, reaction was performed at various temperatures (30^0C to 60^0C) in 50 mM phosphate buffer (pH 7.0). The sudden fall in activity was observed when temperature increased above 35^0C from 70 IU/ml to 28 IU/ml, then there was gradual decrease in enzyme activity upto 60^0C. The optimum temperature of chitinase was measured 35^0C which yielded enzyme activity 70 IU/ml. Similarly the optimum temperature for chitinase was reported for Bacillus firmus by Loni et.al (2011) at 37^0C, for Streptomyces sp. by Balakrishnan et.al (2012) at 30^0C and for Aeromonas hydrophila and Aeromonas punctata was reported by Kuddus et.al (2013) at 37^0C.

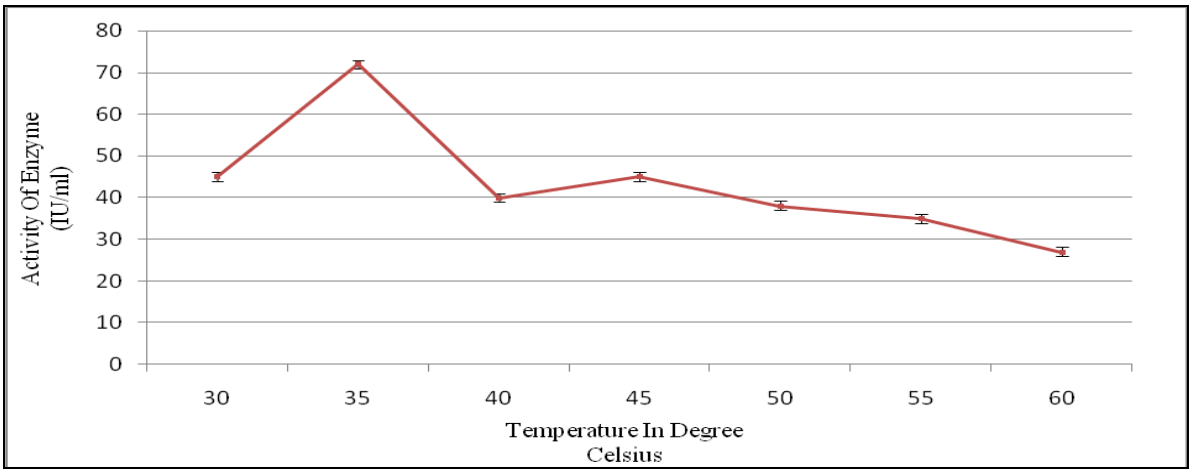

Figure 2: Effect of Temperature on chitinase from *Achromobacter denitrificans* .

3.3 Effect of pH on chitinase activity

Studies of the influence of pH on the enzyme activity were carried out and the results are shown in figure 3. The Chitinase from *Acromobater denitrificans* was active at wide range of pH between 5 to 9. The enzyme activity was moderate up to pH 9 and then there was sudden fall in activity at pH 10. The optimum pH for the enzyme activity was found to be 7.0, below pH 5 their was no significant activity. Similar optimum pH values were also reported for *A. punctata* (Kuddus et.al 2013) and *Streptomyces sp.* (Balakrishnan et.al 2012).

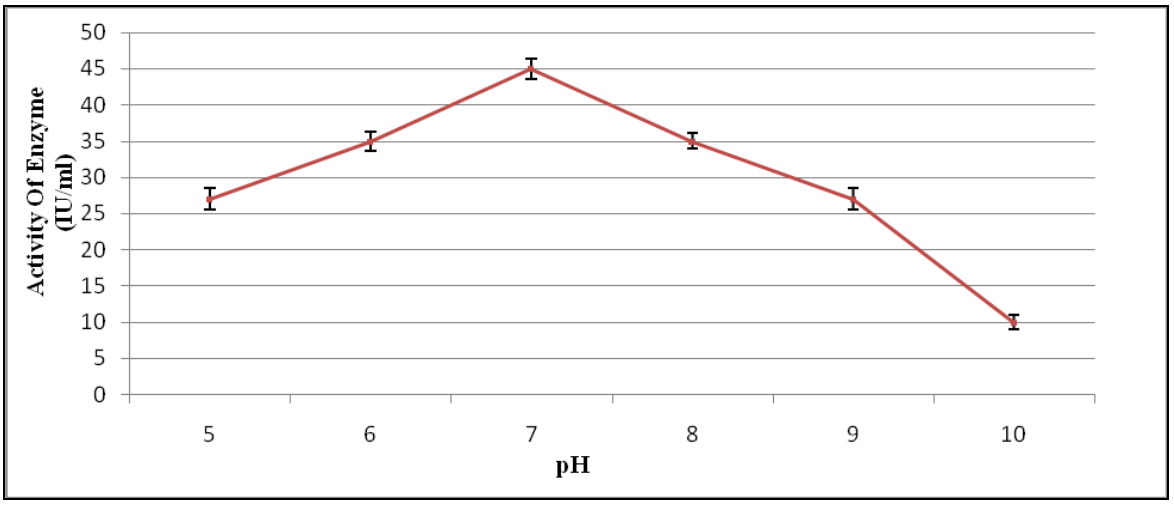

Fig 3: Effect of pH on chitinase from Achromobacter denitrificans.

3.4 Effects of metal ions on chitinase activity

The enzyme showed varying degree of activity at different concentrations of metal ions. The activity of enzyme was stimulated by the addition of metals like $Na^{+,}$ Cu^{++} and Ca^{++} but Hg^{++} showed absolute inhibition, while Zn^{++} showed recognisable inhibition on the enzyme activity. Optimum stimulatory concentration was noted between 0.4mM to 0.8 mM. From figure 4 it may be certainly concluded that all metal ions show inhibitory effect at higher concentrations.

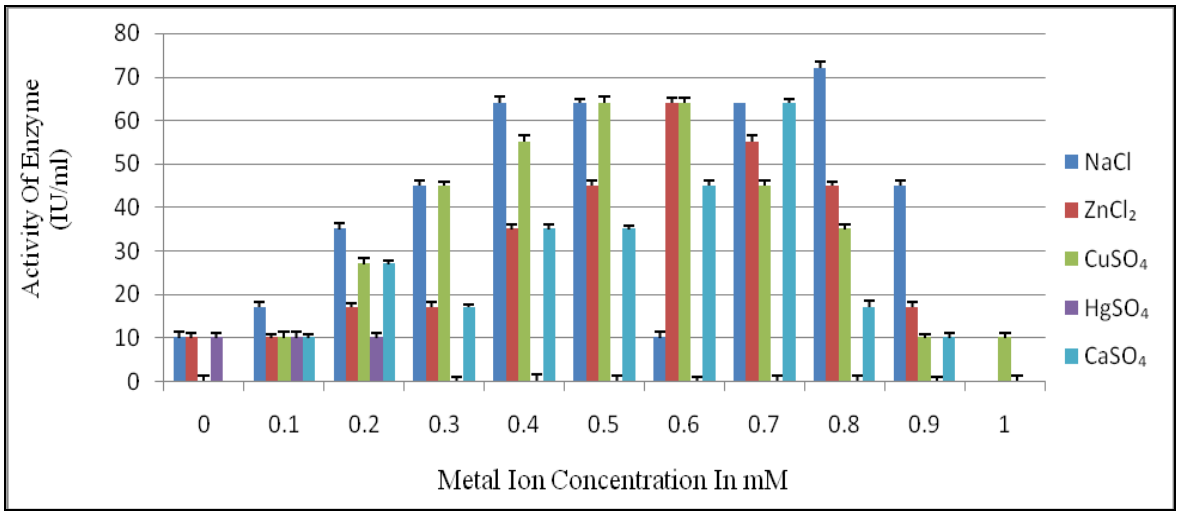

Figure 4: Effect of metal ions conc. on chitinase from *Achromobacter denitrificans* .

3.5 Effect of substrate concentration on chitinase activity

It was noted that there was low activity at low substrate concentration but as substrate concentration goes on increasing, the enzyme activity also increased up to 7mM. After that further increase in substrate concentration did not affect the activity significantly as shown in figure 5.

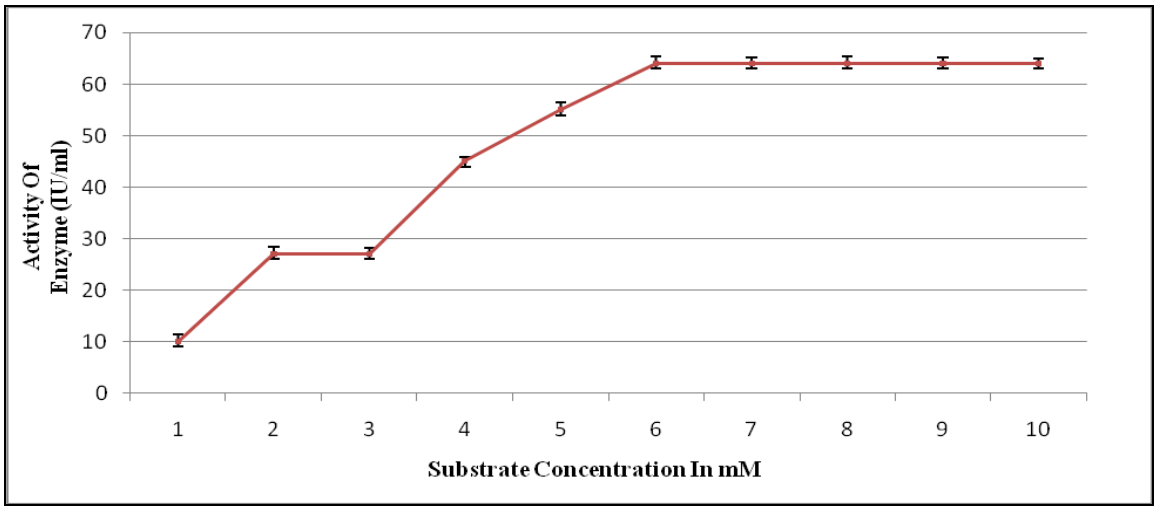

Figure 5: Effect of substrate conc. on chitinase from Achromobacter denitrificans.

IV. Conclusions

This study explores the biodegradation of chitin with purified exo-chitinase enzyme from *Achromobacter denitrificans*. The literature so far studied has no reports for any chitinolytic activity by *Achromobacter denitrificans*, therefore it is for the first time reported to degrade chitin. The optimum temperature and pH for enzyme was found to be 35ºC and 7.0 respectively. In case of metal ions Cu^{++} and Ca^{++} were found to be good activators of enzyme, while Hg^{++} was absolute inhibitory and all metal ions show inhibitory effect at higher concentrations . As substrate concentration increased the enzyme activity also relatively increased up to 7 mM, after which further increase in concentration did not influenced the enzyme activity. As chitin is recalcitrant its degradation by chemical and physical methods are harmful to environment as burning of chitinous material generates carbon dioxide and carbon monoxide gases. Though biodegradation is a slow process but it is ecofriendly. Chitinase enzyme and their products has many applications in industry hence it can be used for sustainable development.

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