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Production of gelatinase enzyme from *Bacillus* spp isolated from the sediment sample of Porto Novo Coastal sites.

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ABSTRACT

Objective: In this study, gelatinase producing bacteria were probed from sediment samples of Porto Novo Coastal sites, India. Screening and identification of potential strain were done followed by optimization of physico-chemical parameters; bulk production and gelatinase extraction were carried out. **Methods:** For probing of gelatinase potential producer primary and secondary screening was carried out for qualitative and quantitative estimation. Optimization of physico-chemical parameters for improved production of gelatinase enzyme and large scale of gelatinase was produced. Gelatinase precipitation was standardized using different saturation rates of ammonium sulphate from 10 to 100% at 4℃.**Results:** There were 8 morphologically different gelatinase producing bacteria were initially delved through primary screening tests. *Bacillus spp* produced maximum gelatinase activity (2.1U/mL) in secondary screening test. Optimizing its abiotic and biotic factors, maximum enzyme activity was achieved at 48h incubation period (2.2U/mL), 2.5 pH (2.5U/mL), 35℃ temperature (2.55U/mL), 0.8% lactose (2.6U/mL), 1.4% gelatin (2.9U/mL) as the ideal carbon source and nitrogen source, 1% salinity (2.9U/mL) and 3ml of inoculum containing 5.6×106/ mL (3.3U/mL). From the optimized factors, bulk production was carried out and saturation rate of 40% ammonium sulphate, precipitated out maximum enzyme with lowered dry weight indicates its enzyme purity and recovered enzyme showed 4.1U/mg of activity. **Conclusion:** The study revealed that the isolated strain *Bacillus spp* has its potentiality for industrial scale production and the results will stand as a base line data for the application of gelatinase in future.

1.Introduction

The biological and chemical diversity of the marine environment has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, enzymes, fine chemicals, and agrichemicals[1]. An enzyme from the marine source may be a unique protein molecule not found in any terrestrial organism or it may be a known enzyme from a terrestrial source but with novel properties[2]. Enzymes reported till date is produced mostly by microorganisms of terrestrial origin. However, only a few number of reports reported from marine producers, their capability have not been explored in details hence the present study.

There were so many chemicals persist in an environment, THB (Total heterotrophic bacteria) populations adopt to produce various enzymes including gelatinase to decompose and recycle it. In this backup an attempt has made to probe for potential gelatinase bacterial producer which makes a promising strain with desired nature for industrial large scale production. Gelatinase is one type of diverse group protease, an extracellular metallo- endopeptidase or metalloproteinase which is able to hydrolyze gelatin and other compounds such as pheromone, collagen, casein and fibrinogen[3,4]. Gelatinase and Collagenase are important metalloproteases and these enzymes are widely used not only in chemical and medical industries but also in food and basic biological science[5]. Gelatinase enzyme produced by microorganism hydrolyze gelatin into its sub-

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compounds (polypeptides, peptides and amino acids) that can cross the cell membrane and be used by the organism. Forms of gelatinases are expressed in several bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus, Clostridium perfringens* and *Serratia marcescens*.

In humans, gelatinase are matrix metalloproteinase (MMP 2 and 9) are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. These enzymes even participate in the turnover of extracellular matrix and together the MMPs are able to degrade any of the matrix components[6].

Nowadays gelatinase have received considerable attention as targets for drug development because of their potential role in connective tissue degradation associated with tumor metastasis[7,8]. The potential uses of gelatinase and their high demand, the need exists for the discovery of new strains of bacteria that produce enzymes with novel properties and the development of low cost industrial medium and extraction formulations.

In our present study, a potent gelatinase producer *Bacillus spp* isolated from sediment sample Porto Novo coastal sites, India. Discovering such new species, producing gelatinase with novel properties will be of great value to the enzyme and pharmaceutical industry for different applications. The isolated strain was optimized against various media components and fermentation conditions for improved and cost-efficient enzyme production. Extracellular enzyme production is highly influenced by media variation in components such as C/N ratio[9, 10]. Besides this, several other factors, such as inoculum density, pH, temperature and incubation time, also affect the amount of enzyme produced[11]. In our study we produced gelatinase enzyme from a potential wild strain *Bacillus spp* with maximum activity and the results showed its applicability for the industrial scale production of this enzyme for various commercial applications.

Materials and Methods

2.1 Collection of sediment samples

Sediment samples were collected from five different off shore sites of Porto Novo coastal region, Cuddalore district, Tamilnadu, India using a sterile spatula. The central portion of the collected samples were aseptically transferred to a sterile polythene bags and transferred to the laboratory in an ice box maintained at 4o C for further study.

2.2 Isolation of Gelatinase producing bacteria

The collected sediment samples were serially diluted and spread plated on gelatin agar containing gelatin -15.0g, peptone - 4.0g, yeast extract - 1.0g, agar - 15.0g, 50% aged sea water - 1000ml and incubated for 48hrs at 37℃.

2.2.1 Detection of gelatinolytic bacteria (Primary screening)

After incubation period, bacterial colonies were observed on gelatin agar plate. The colonies were replica plated into a fresh gelatin agar plate, after incubation period the replica plate was examined for gelatinase producer using 15% mercuric chloride in 20% (vol/vol) concentrated HCl solution [12]used as protein precipitating agent. From the mother gelatin agar plate the gelatinolytic bacterial colonies were collected for further study.

Gelatin liquefaction test

Gelatin liquefaction (the formation of a liquid) was tested by stabbing gelatin agar (semisolid with 7.5g/L agar) deep tubes. After 48h of incubation, the cultures placed in a refrigerator at 4 ℃ until the bottom resolidifies. If gelatin was hydrolyzed, the medium will remain liquid after refrigeration. If gelatin was not hydrolyzed, the medium will resolidify during the time it is in the refrigerator. The strains liquefied gelatin was taken for quantitative study.

2.2.2 Potential strain selection by enzyme assay (Secondary screening)

Gelatinase activity was quantitative tested according to Tran and Nagano[13]. The reaction mixture contained 0.3 ml of (0.2%) gelatin in water, 0.2 ml of (150 mM) Tris-HCl, pH 7.5, containing 12 mM CaCl2, and 0.1 ml sample (crude enzyme). The reaction mixture was incubated at 30℃ for 30 min and stopped by the addition of 0.6 ml of (0.1 N) HCl. The released free amino group's amount was measured by the Ninhydrin method. Gelatinase activity is expressed as μmol of leucine equivalent per min/ml of the culture filtrate (Hamza *et al*, 2006). The same mixture except gelatin was used as blank.

2.3 Extracellular enzyme.

The potential bacterial strain was freshly inoculated in 100ml gelatin broth and incubated for 48h incubation at 37 ℃. A portion of 50ml was taken from the cultured broth and centrifuged at 3000rpm for 20 min; pellet and supernatant were collected separately. The cell pellet was dissolved in 50ml phosphate buffer and sonicated for 1min. Remaining portion of 50ml cultured broth was sonicated for 1min without centrifugation. The three different mediums supernautant, sonicated cell pellet and sonicated broth culture were tested by enzyme assay for gelatinase enzyme location whether it is extracellular or intracellular or located at both sites.

2.4 Strain identification

The pure cultures of gelatinase producing bacteria are needed to be identified. Identification was done with Biochemical methods with the help of Bergy's Manual of Determinative Bacteriology.

2.5 Optimization of physico chemical parameters for maximum gelatinase production.

The selection of the best medium components and their concentrations plays an important role in product development, not only the media component but also the environmental parameters like pH, temperature and other things also has its role in it.

The experiments were done by adopting search technique i.e., varying parameters one at a time, were conducted in 250ml Erlenmeyer flask containing gelatinase production medium and all the experiments were carried out in triplicate and the average values were calculated. The range of parameter achieved by one step was fixed in subsequent experiments.

Optimization was done with the base of a basal medium, the composition of the basal medium is (g/L): glucose- 2.0; gelatin- 0.5; peptone- 0.5; yeast extract- 0.5, and salt solution- 50 mL (salt solution containing [g/L]: KH2PO4,-5.0; MgSO4.7H2O - 5.0, and FeSO4.7H2O- 0.1] with a pH of 7.0 and incubated at 37℃.

2.5.1 Incubation period

Incubation period was carried out ranging from 8h to 96h with 8h interval and the gelatinase production was estimated.

2.5.2 pH

The pH of the medium was optimized between the ranges 4 to 9 with intervals of 0.5 for maximum gelatinase production.

2.5.3 Temperature

The effect of temperature on gelatinase production was estimated between 20℃ to 50℃ with the interval of 5℃.

2.5.4 Carbon sources

The influence of different carbon source like glucose, fructose, lactose, maltose, starch, with varying concentration from 0.1% to 1% with 0.1% interval were tested for maximum gelatinase production.

2.5.5 Substrate concentration

Influence of enzyme substrate gelatin ranging from 0.1% to 1% with an interval of 0.1% was tested.

2.5.6 Salinity

The effect of varying salt concentration was checked between 5 - 50 ppt with the interval of 5 ppt since the strain is of marine origin.

2.5.7 Inoculum density

Varying concentration of inoculum density with respect to gelatinase production was tested using varying concentrations of 1ml to 5ml cultured broth inoculation containing 5.6×106/ ml to 100ml of media.

2.6 Inoculum preparation

Potential bacterial strain was cultured using optimized production medium in 30ml broth of 100ml Erlenmeyer's conical flask and incubated for 48hrs at 35℃.

2.7 Bulk production.

For mass scale production, 1000ml of optimized gelatinase production medium was prepared in 2 litre Erlenmeyer's conical flask. After sterilization, optimized quantity of prepared inoculum concentration was added and incubated by optimized conditions. Finally the medium was tested for its gelatinase production under aseptic conditions in regular intervals.

2.8 Ammonium sulphate fractionation and partial purification.

Protein precipitation was carried out with different concentration of ammonium sulphate from 10 to 100% saturation level at 40C unless as described[14]. After optimized incubation period, the broth was sonicated for 1 min and the resulting medium was centrifuged at 3000rpm for 20min. The supernatant was collected and precipitated with different saturation rates using ammonium sulphate and kept at over night. The precipitated proteins were collected using centrifugation at 10000rpm for 20 min and the pellets were collected. The protein solution was dialyzed using dialysis membrane against the phosphate buffer solution (PBS) at pH 7 for 24hr, with intermediate changes of PBS to remove the salt and the final solution was subjected to lyophilize. The final lyophilized sample was gelatinase assayed and the dry weight of the sample was also taken into account for selection of optimized saturation level for fractionating desired protein.

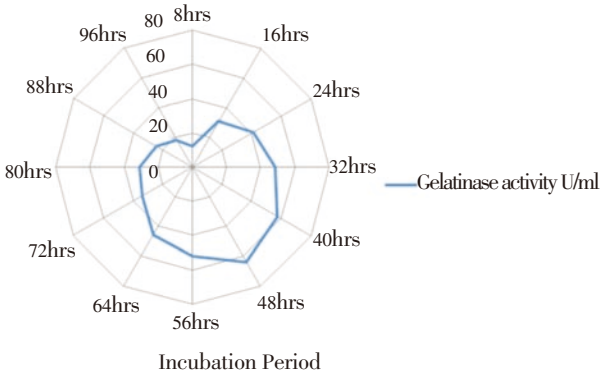
3. Result

In our study, sediment samples were collected from five different offshore coastal sites of Porto Novo. The samples were taken aseptically from the sampling site transferred and processed immediately in the lab for the isolation of gelatinase producing bacteria. The samples were serially diluted and spread plated on gelatin agar. After 48h of incubation period, sediment samples possessed more gelatinase producing bacterial density between 2.3×102 to 1.7×103 CFU.

Figure 1. Bacillus spp showing gelatinase activity against HgCl2 solution in gelatin agar

The plate was used for further analysis are needed to be replica plated, after incubation period the replica plate was flooded with mercuric chloride solution. While doing this, the proteins present in the plate were precipitated and showed an opaque appearance on the proteineous sites if the bacterial colonies produced gelatinase enzyme showed

a clear zone around the colonies indicates the hydrolysis of gelatin substrate and the bacterial colonies were taken from the mother plate for further analysis. There are 7 different bacteria showed zone around their colonies in replica plate were taken from the mother plate was needed to be repeatedly pure cultured on gelatin agar plate. After achieving the axenic culture, the bacteria were broth cultured, centrifuged and the pellets were lyophilized. Lyophilize preserved bacterial cells were used for further study. The gelatinase producing bacteria isolated in our study were once second checked for gelatinase production using gelatin liquefaction test, all the 7 isolated bacteria liquefied gelatin stab agar under refrigeration of 4℃ showed the synthesis of gelatinase enzyme.



Figrue 2. Effect of different incubation period on gelatinase production

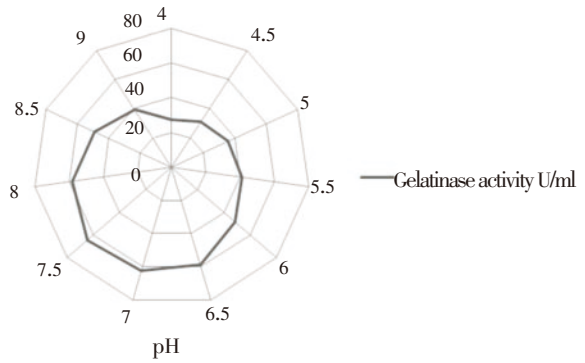


Figure 3. Effect of different pH on gelatinase production

The potential bacterium was selected based on the results obtained from zone measurement of axenic cultures on the gelatin agar (fig.1) and enzyme quantitative assay. Out of which, strain no P7 produced maximum gelatinase activity of 2.1 U/ml and maximum zone formation was selected as potential gelatinase producing bacteria. P7 was identified as *Bacillus spp* with the help of Bergy's Manual of determinative bacteriology. The location of enzyme plays an important role in the down streaming process whether the enzyme located extracellular or intracellular or on both sites. The cell free supernatant, sonicated cell pellet and supernatant of sonicated broth cultures were subjected into studies and it was screened by quantitative enzyme assay. Supernatant of sonicated broth culture showed maximum activity of 2.1 U/ml followed by cell free supernatant 1.7U/ml and sonicated cell pellet 0.5U/ml, it was clearly stated that the gelatinase enzyme was located at both the sites but maximum of it was located extracellular.

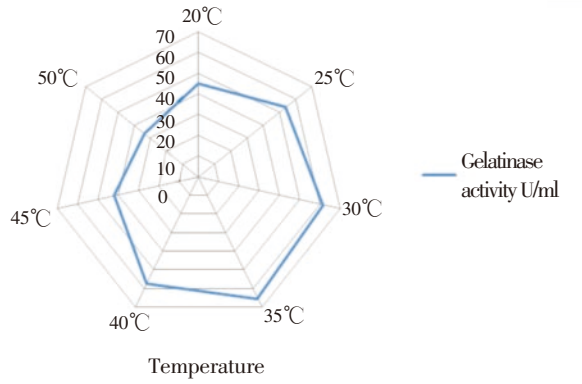


Figure 4. Effect of different temperature on gelatinase production

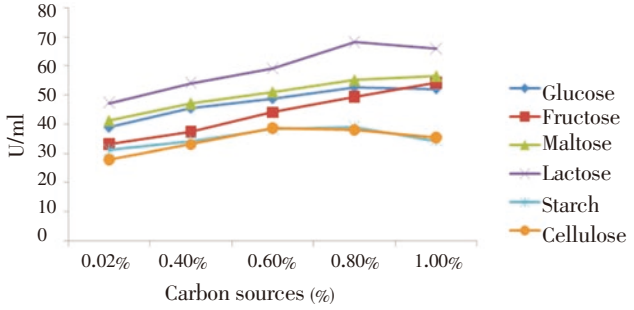


Figure 5. Effect of different carbon sources with varied concentration on gelatinase production

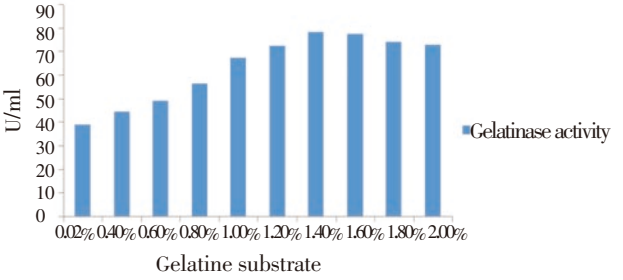


Figure 6. Effect of different concentration of gelatin on gelatinase production

For the maximum production of gelatinase enzyme, *Bacillus spp* was optimized for various physic-chemical parameters, initially the optimal incubation period was examined from 8h to 96h (fig.2) and gelatinase enzyme production was maximum at 48h (2.2U/ml). The pH of the medium was adjusted between 4 to 9 with 0.5 varying intervals (fig.3), the optimum pH for maximum gelatinase production was at 7.5(2.5U/ml). The effect of temperature on the enzyme production was found out (fig.4), showed maximum gelatinase production at 35℃ (2.55U/ml). Optimization of various carbon sources (fig.5) showed lactose as the best source of 0.8% producing 2.6U/ml gelatinase activity. The present study revealed that gelatin the enzyme substrate concentration plays an important role in the enzyme production showed 1.4% of concentration produced 2.9U/ml of yielding (fig.6). Gelatin also acts as a best nitrogen source among the tested sources for the maximum growth of the *Bacillus spp*. As the sample collected from estuary, it is the place for both fresh and marine environment

habitat so salinity also might plays an important role for maximizing gelatinase production. Salinity concentration of 10ppt produced 2.9U/ml which showed maximum activity and the absence of salinity showed retarded growth of the organism as well as gelatinase production (fig.7). Finally the inoculum density was needed to be optimized for estimating the correct quantity of inoculum for better production of gelatinase enzyme (fig.8). It was estimated that 3ml of inoculum containing 5.6×106/ ml microbial density per 100ml of broth attained 3.3U/ml.

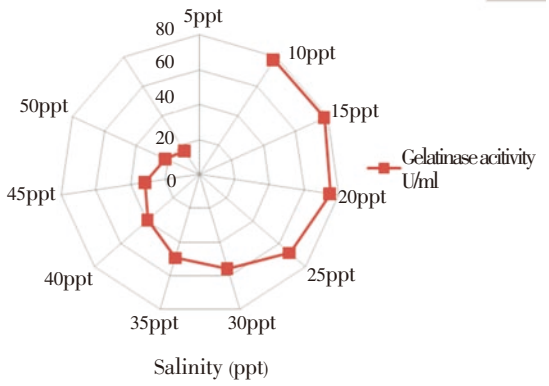


Figure 7. Effect of different salinity on gelatinase production

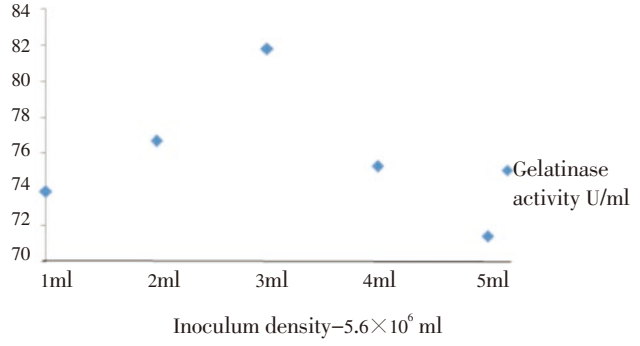


Figure 8. Effect of different inoculums density on gelatinase production.

Based on this optimization results obtained, 30ml of inoculum was prepared with optimized physico chemical parameters and inoculated with isolated potential bacterium *Bacillus spp*. The inoculum was transferred to already sterilized 1000ml bulk media on 2 liter Erlenmeyer's conical flask which was incubated for 48h. In mass scale culture the strain produced 3.6 U/ml of gelatinase.

On down streaming process the broth cultures were aseptically sonicated using ultrasonicator for one minute and it was centrifuged at 3000rpm for 20min. Resulting supernatant was collected aseptically and it was divided into 10 batches each containing 100ml. Each batch was added with different saturation rate of ammonium sulphate from 10 to 100%. The batches were kept at overnight for protein precipitation and centrifuged for 20min at 10,000rpm. The pellets containing the precipitated proteins were lyophilized and dry weight of the different saturated proteins were estimated (tab.1). The saturation rate of 40% ammonium sulphate showed maximum gelatinase activity of 4.1units/mg with lower dry weight of crude protein after precipitation. The study revealed that the isolated strain *Bacillus spp* has its potentiality for industrial scale production and precipitated enzyme has stable and showed reproducible results over consecutive tests. The results will stand as a base line data for the application of gelatinase in future.

Table 1

Crude gelatinase enzyme precipitation using different saturation rates of ammonium sulphate.

Percentage saturation rate of ammonium sulphate	Dry weight of crude enzyme per liter of cell free supernatant	Enzyme activity
10	0.17	1.7units/mg
20	0.33	2.3units/mg
30	0.41	3.5units/mg
40	0.63	4.1units/mg
50	0.72	4.1units/mg
60	0.78	3.9units/mg
70	0.89	2.7units/mg
80	1.01	2.3units/mg
90	1.17	2.1units/mg
100	1.19	1.7units/mg

Discussion

In the present study an attempt was made to isolate wild type bacterial strains produce gelatinase enzyme and optimize the various limiting and maximizing factors of the isolated strain *Bacillus spp* from the sediment sample of offshore coastal site, Porto Novo, Tamil Nadu. In our study, the above results clearly showed that the sediment sample has large number of bacteria which might be due to richness of nutrients in coastal sediment.

The gelatinase bacterial producers were screened by gelatin agar with the help of mercuric chloride solution and isolated colonies were selected based on the distinct morphology. The selected bacterial colonies were pure cultured and individually tested for its enzyme production. Based on the results obtained from primary screening methods seven bacterial strains were showed good results and they are named as P1 – P7 (P – Porto Novo). P7 produced maximum gelatinase activity over the rest of the other six strains and it was identified as *Bacillus spp* with the help of Bergy's Manual of determinative bacteriology.

When optimizing the physico-chemical parameters of potential bacteria *Bacillus spp* for maximum gelatinase production, initially the optimal incubation period was examined at 48h (2.2U/ml). Similar[15]result was observed as 48h was the ideal incubation period for protease production using *B. subtilis* PE-11. One major factor that is pH may constitute the maximum production of gelatinase production achieved at pH 7.5. However[16]the maximal protease yield was obtained from the culture *Strptomyces pseudogrisiolus* NRC-15 at pH 9.0. Different temperature ranges were checked for the maximum gelatinase production on the enzyme production was found out (fig.3), showed maximum gelatinase production at 35℃ (2.55U/ml), similar result was observed[17]using *Bacillus halodurans* showed maximum activity at 37℃ with protease enzyme production. In our study optimization of various carbon sources showed lactose

as the best source of 0.8% producing 2.6U/ml gelatinase activity, a study[18]described lactose did not influence significantly the production of gelatinase using *Enterococcus faecalis*. The present study revealed that gelatin the substrate concentration of 1.4% produced 78.5U/ml yielding maximum. The enzyme substrate itself acts as the best nitrogen source and it can increases both the growth and enzyme production of the bacteria. However Shaheen *et al*[19]reported that soyabean and casein proved as the best nitrogen sources for protease production. As the sample collected from seashore area salinity also might plays an important role for maximizing and limiting gelatinase production. Salinity concentration of 10ppt produced maximum activity of 2.9U/ml. Beyond the limit showed limited growth of the cell thereby minimal gelatinase production and the results clearly showed that the isolated strain belongs to the obligate marine species.

Mass scale production of gelatinase enzyme was achieved with the optimized factors and standardized for ammonium sulphate precipitation showed 40% using 0℃ was the ideal condition precipitated out maximum gelatinase enzyme having 4.1U/mg. The increasing concentration of ammonium sulphate beyond 40% precipitated out maximum dry weight of protein but may denature the desired protein gelatinase enzyme and below the required concentration precipitate very less quantity of gelatinase enzyme. However a study revealed[20]gelatinase enzyme with 60% ammonium sulphate showed maximum activity of 3.5 U/mg. From our study the enzyme gelatinase was not only bulk produced it was also extracted with simple and cheaper method using ammonium sulphate having a good reproducible results for commercial scale gelatinase enzyme production.

Conflict of interest statement

We declare that we have no conflict of interest.

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