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# Production of Pullulan using Jaggery as substrate by *Aureobasidium pullulans* MTCC 2195

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**Abstract**

Shake-flask fermentation, under batch cultivation, was investigated for the production of fungal exopolysaccharide, pullulan using jaggery (a traditional concentrated sugar cane juice) as a carbon substrate by *Aureobasidium pullulans* MTCC 2195. Change in the initial pH (from 3.0 to 7.0) of media containing jaggery was varied to study the effect of pH in the fermentation and maximum pullulan yield was obtained at a pH of 5.0. An increase in the initial concentrations (50, 75, 100 g/L) of jaggery in the media produced the maximum pullulan content as 21.6, 19.7 and 18.6 g per 100 g of jaggery, respectively, used. A sucrose based defined media were also used for comparison purposes. Fourier Transform InfraRed (FTIR) spectroscopic analysis was done to confirm the functional groups of synthesized pullulan and compared with that of commercial pullulan.

**Keywords :** Jaggery, Pullulan, pH, FTIR, *Aureobasidium pullulans* MTCC 2195

## Introduction

The recent trends in cost-effective production of microbial polysaccharides, cheaper substrates are being used to meet the economic advantage of production. One of such is fungal Exopolysaccharide (EPS) called pullulan owing to its excellent properties, became potential compound in several high-value technological platforms like food, biomedical, pharmaceutical, film and packaging industries (1-4).

Pullulan, a water-soluble, neutral, linear, homo-polysaccharide mostly produced by a ubiquitous black, yeast-like fungus, *Aureobasidium pullulans*. Pullulan, regarded as GRAS (Generally Recognized As Safe, by USFDA), composed of maltotriose repeating units connected by $\alpha$-1, 6 linkages (3 glucose moieties in maltotriose joined by $\alpha$-1, 4 glycosidic bonds). However, the fermentative production of pullulan in submerged conditions on wide variety of agro-industrial residues and wastes as low-cost substrates, were attempted by many researchers (Table 1) (5-13). In earlier studies, *jaggery* (also known as *Panela* or *gur*), a traditional non-centrifuged, un-purified and concentrated sugar cane product, from cottage industries, contains 75-85% sucrose was used for production of pullulan by other strains of *Aureobasidium pullulans* (5, 13).

In this present study, a medium composed of *jaggery* as a carbon source for pullulan production by *Aureobasidium pullulans* MTCC 2195 was attempted. The specific aims were to vary the initial concentrations of *jaggery*, initial pH and to determine the kinetics of pullulan fermentation. Characterization of produced pullulan was also performed to confirm the results.

## Materials and Methods

***Materials Used:*** Jaggery was purchased from the local market and found to be mainly composed of sucrose. The composition of Standard Cultivation Medium (SCM) used in the shake-flask fermentation (in g/L) is sucrose, 50.0; yeast extract, 3.0; $KH_2PO4$, 5.0; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 1.0 and distilled water 1 L. All the chemicals were purchased from Qualigens Chemicals and the biochemicals are purchased from M/s. HIMEDIA chemicals Ltd. Standard pullulan was purchased from M/s. Kumar Organics Pvt. Ltd., Bengaluru, and was used in structural characterization.

***Micro-organism and Inoculum Development:*** Micro-organism used in this study, *Aureobasidium pullulans* MTCC 2195, was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh and maintained on potato dextrose agar (PDA) slants at $4^0$C and subcultured prior to each experimental run. A loop-full of freshly grown cultures from PDA agar slants were transferred to a 250 ml conical flask containing 50 ml Standard cultivation media. Media pH (initially) was adjusted to 5.0 (after autoclaving at $121^0$C, for 15 min.) and incubated at $30^0$C for 48h on a rotary shaker at 150 rpm. This resulted suspension (at 5% v/v) was then used as inoculum for *jaggery* medium fermentations.

***Shake flask fermentation:*** Shake flask fermentations were carried out with Standard Cultivation Medium components at initial sucrose concentrations (g/L) of 50, 75 and 100, individually. Further, the cultivation media (JCM) was made by replacing sucrose with jaggery (based on weight but not on % of sucrose content) for the same concentrations (g/L) of 50, 75 and 100. Both SCM and JCM in 100 ml aliquots were distributed in 500 ml Erlenmeyer flasks and autoclaved. These sterilized media were inoculated, 5% (v/v) aseptically and incubated for 172 hours at $30^0$C and 150 rpm on a rotary shaker. Fermentation broth samples were collected, aseptically, at irregular intervals and determined the concentrations of dry cell biomass, pullulan and residual sugar.

***Effect of initial pH on fermentation:*** In order to study the influence of pH on the shake flask fermentation for the pullulan production, the pH of *jaggery* in cultivation medium (after autoclaving) was adjusted to 3.0, 4.0, 5.0, 6.0 and 7.0, individually, using either 1N HCl or 1N NaOH and left uncontrolled during the fermentation. A 100 ml sterile media in 500 ml Erlenmeyer flasks by inoculating 5% (v/v) inoculum were incubated for 156 h at $30^0$C and 150 rpm on a rotary shaker. The samples were withdrawn for every 12 hours and analysed for cell biomass, Pullulan and residual sugar contents.

***Estimation of dry cell biomass and pullulan:*** At specific intervals of time, 2 ml of broth volume from each flask was centrifuged at 10,000 rpm for 20 min at ambient temperature to separate the cell biomass (pellet) from supernatant liquid. The collected cell biomass was washed twice with saline and distilled water and dried to constant weight in an oven at $90^0$C. The dry cell biomass weight was expressed in g/L. The polysaccharide, pullulan, was precipitated (kept at $4^0$C for 12 hours) using cell-free supernatant liquid by adding cold ethanol (in the ratio of 1:2 v/v). The precipitate obtained was filtered through a pre-weighed Whatman No.1 filter paper and dried to constant weight at $80^0$C. The dry weight of pullulan and yield of pullulan were expressed in g/L and gram EPS per 100 g of sugar consumed.

***Estimation of residual sugar concentration:*** The residual sugar content in the cell-free fermentation broth was measured by the Miller's method (14) using double beam ELICO SL-159 UV-Visible Spectrophotometer.

***Characterization of pullulan by FTIR spectroscopy:*** The precipitated pullulan from each flask, at the end of fermentation was characterized to compare the quality of pullulan obtained from sucrose and *jaggery* media. The

structural characterization of pullulan was carried out using Fourier Transform InfraRed (FTIR) spectroscopy and IR spectra were recorded with Shimadzu IRTracer-100 Fourier Transform Infrared (FTIR) Spectrophotometer.

## Results and Discussion

***Effect of initial pH on fermentative production of pullulan in jaggery medium:*** Effect of environmental variables like pH of the cultivation medium enhanced the yields of pullulan formation by *A. pullulans* on wide variety of carbon substrates (5-13, 15-19). Changes in initial pH of fermentation medium affects the growth rate of *Aureobasidium pullulans* MTCC 2195 using coconut by-products, bakery waste, cassava and maize powders, cashew fruit juice, were reported by Thirumavalavan *et al*. (9, 10). In another study, Vijayendra et al. (5) varied the initial pH from 2 to 7 for the study of *A.pullulans* CFR-77 growth and reported that maximum pullulan production was achieved at an initial pH of 5.0. Here, we attempted the change in initial pH (3.0 to 7.0) effect on the kinetics of *A.pullulans* MTCC 2195 in jaggery medium. Final (at the end of 156 h) pullulan and biomass concentration (g/L) as a function of pH is shown in Fig. 1. Pullulan content released into the medium increases with pH up to 5.0 and then decreased. The maximum concentration (9.88 g/L) of pullulan exopolysaccharide was obtained at pH 5.0.

***Study of jaggery as a carbon source on growth of A. pullulans and pullulan production:*** Preliminary checking of *Jaggery* in replace of sucrose to study the growth of *Aureobasidium pullulans* MTCC 2195 in the cultivation media with agar on petri plates was performed. Several workers have exercised the wide variety of carbon substrates, ranging from agro-industrial residues and wastes for the economic production of Pullulan and the results are summarized in Table 1. The earlier investigations (5, 13) on *jaggery* reveal that *jaggery* could be used as a good carbon source, because of its high sucrose%, for the growth of *Aureobasidium pullulans* and towards cost-effective production of pullulan. S.V.N. Vijayendra et al. (5) reported that the yield of pullulan produced by *A. pullulans* depends on initial sugar concentration in the cultivation medium. So, in the present study the increasing initial levels of *jaggery* concentrations, 50 g/L, 75 g/L and 100 g/L were employed to determine the maximum pullulan content using *A. pullulans* MTCC 2195.

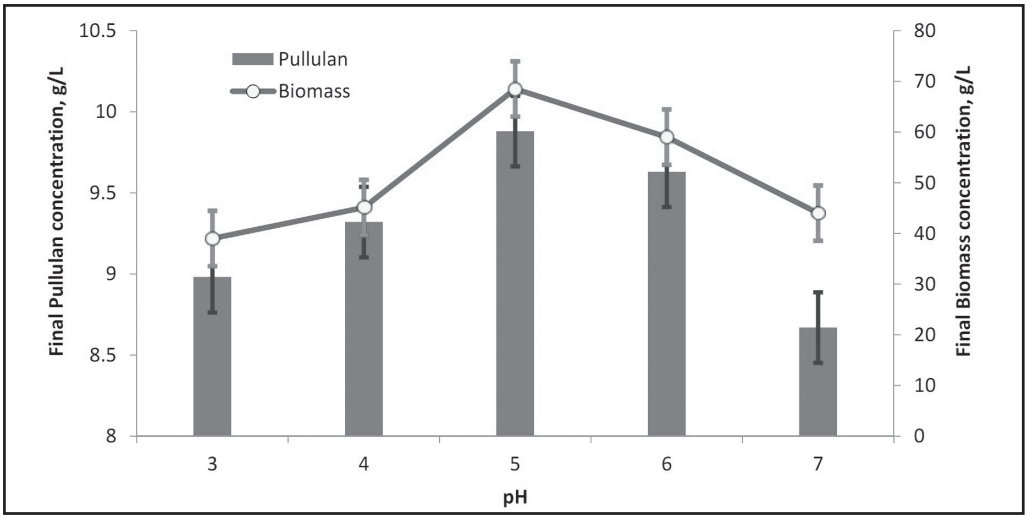


**Fig. 1.** Influence of pH on pullulan and biomass yield using jaggery as carbon source using *Aureobasidium pullulans* MTCC 2195 (error bars indicated).

Fig. 2 (a, b, c) shows the time course profiles of substrate, biomass and pullulan in shake-flask fermentations with increasing initial concentrations, 50, 75, 100 g/L of *jaggery* in the media, respectively. A bar diagram, Fig. 2 (d) indicates the pullulan concentration at specific time points in the above profiles. In all the plots, a considerable amount of sugar in the media was depleted in the early hours of fermentation and results a quick growth of *A.pullulans* and there was a concomitant increase in the pullulan production with decline in the residual sugar content in the media. The maximum pullulan concentrations obtained from *jaggery* medium containing 50, 75 and 100 g/L of sugar concentration were 10.8, 14.8 and 18.6 g/L, respectively.

Fig. 3 (a, b, c) shows the time course profiles of substrate, biomass and pullulan, in case of sucrose containing media with the similar initial sucrose concentrations. A bar diagram, Fig. 3 (d) indicates the pullulan concentration at specific time points in the above profiles. Comparative of *jaggery* and sucrose as carbon substrates for the production of pullulan in this study with literature, is listed in Table-2. Further, kinetic models fit the results of comprehensive biomass growth and pullulan formation and there by predicts kinetic parameters, in a good manner (9, 20, 21). Of these models, Logistic (L), Leudeking- Piret (LP), Logistic incorporated Leudeking-Piret (LILP), Modified Leudeking-Piret (MLP) and Logistic incorporated Modified Leudeking-Piret (LMLP) were tested to describe biomass, pullulan and sugar profiles in the batch cultivation of *Aureobasidium pullulans* (21).

***Structural characterization of pullulan by FTIR spectroscopy:*** Structural characterization

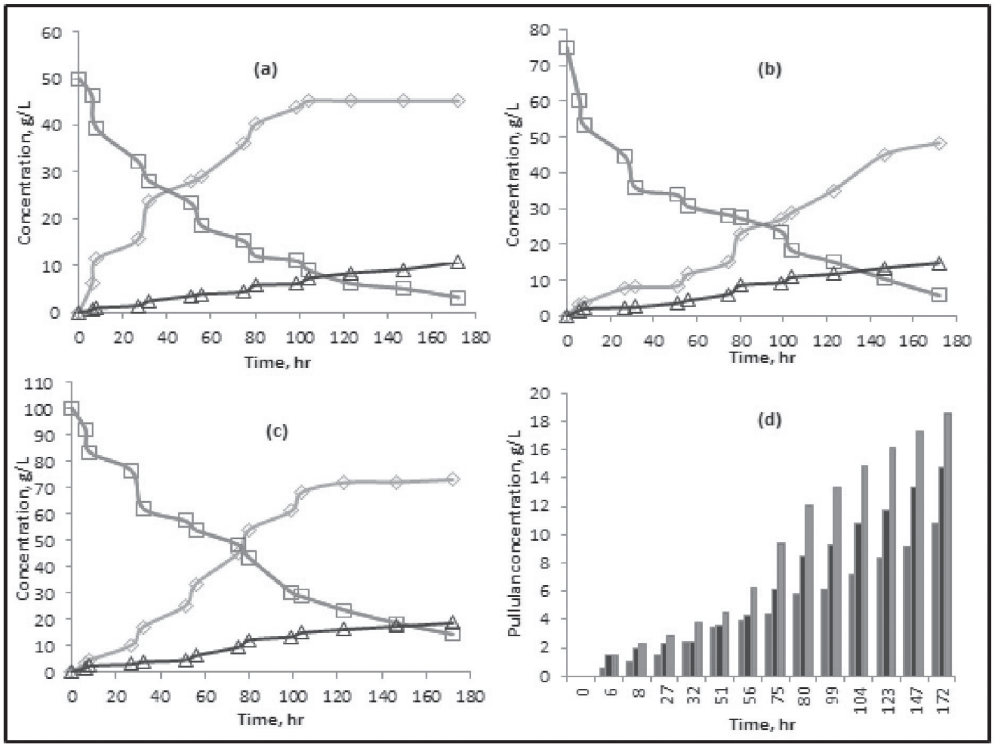


**Fig. 2.** Time course for batch fermentation of *A. pullulans* MTCC 2195 in (a) 50 g/L (b) 75 g/L (c) 100 g/L *jaggery* media: cell dry weight (Ê%), pullulan dry weight (Ä) and sucrose concentration (¡%); (d): variation of pullulan concentration with time in (a), (b),(c) profiles.

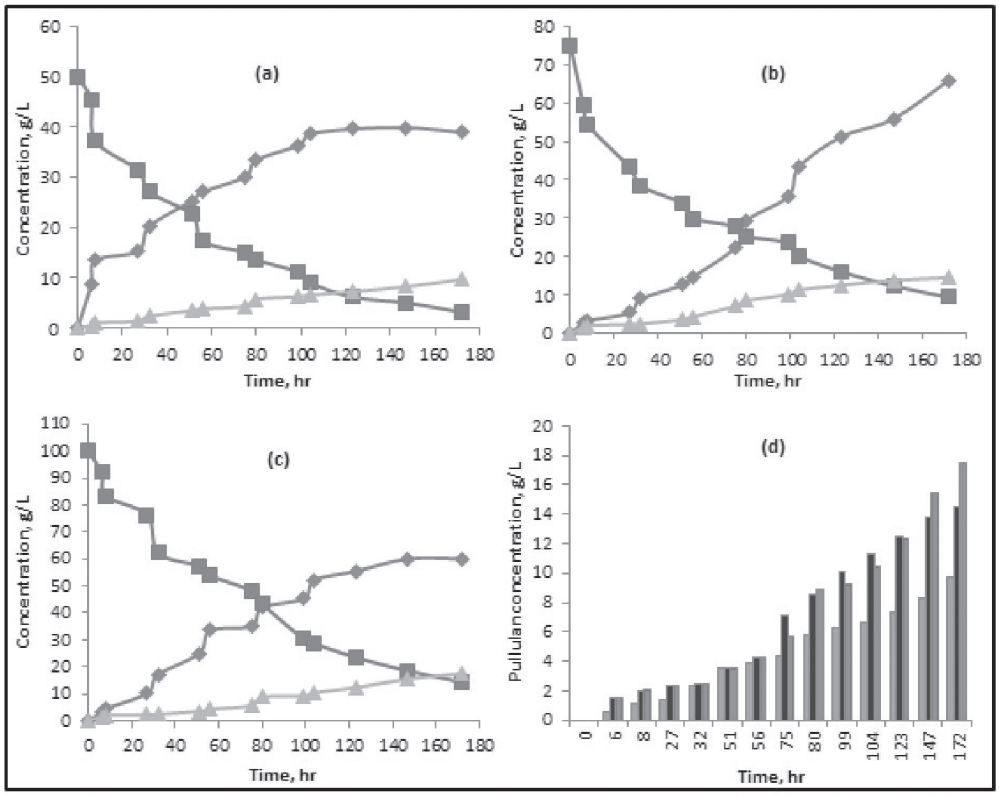


**Fig. 3.** Time course for batch fermentation of *A. pullulans* MTCC 2195 in (a) 50 g/L (b) 75 g/L (c) 100 g/L sucrose media: cell dry weight (Ê%), pullulan dry weight (Ä) and sucrose concentration (¡%); (d): variation of pullulan concentration with time in (a), (b),(c) profiles.

to examine the possible functional groups for commercial pullulan and synthesized pullulan from batch cultivation were done by Fourier Transform InfraRed Spectrophotometer and spectra were shown in figure 4. Top spectrum shows the absorption peaks of standard pullulan and bottom spectrum indicates that of synthesized pullulan from this study. A strong absorption peaks at ranges of 3300 $cm^{-1}$ and 1640 $cm^{-1}$ confirms repeating units of –OH and O-C-O, respectively. In the specific area (1500-650 $cm^{-1}$) is a characteristic of pullulan molecule as a whole. Another strong absorption at 1000 $cm^{-1}$ characterizes the C-O group and peaks at 900 and 650 $cm^{-1}$ proves the presence of $\alpha$-1, 6- and $\alpha$-D-glucopiranosid units, respectively. The similar spectra and frequencies were also observed in other works (6, 8, 22, 23).

## Conclusion

The work attempted, here, first focuses the suitability of using *jaggery* as an effective and cheaper carbon substrate for the maximum production of exopolysaccharide, pullulan by *Aureobasidium pullulans* MTCC 2195. Then, the effect of initial *jaggery* concentration and pH in batch cultivation of polymer production was studied. The results obtained from *jaggery* media were compared to sucrose media and rates of *jaggery* utilization and pullulan production was high, when the initial sugar concentration was comparatively low. The FTIR analysis of synthesized pullulan was also performed to confirm the functional groups in it. All the above results indicate that *jaggery*, an agro-industrial residue, as alternative for carbon source for melanin-free pullulan production. Further, to

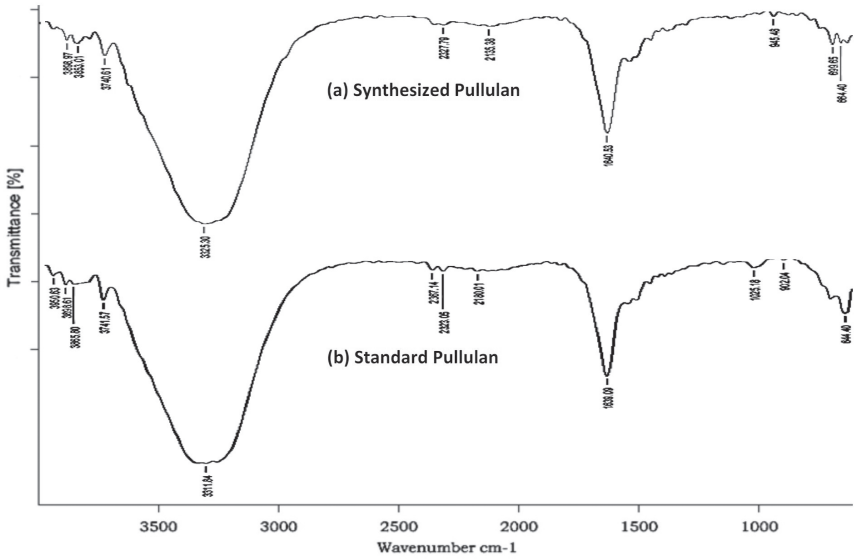


**Fig. 4 (a).** FTIR spectrum of pullulan synthesised in this work (b) FTIR spectrum of standard ullulan commercially purchased.

**Table 1.** Survey on *Aureobasidium pullulans* variants producing Pullulan using different agro-industrial wastes/ residues as substrates, fermentation conditions and yields

| Organism | Type of Carbon Substrate pH | Fermentation conditions Time, hr | | Pullulan content,g/L | Reference |
|---|---|---|---|---|---|
| *A. pullulans* CFR-77 | *Jaggery* | 5.0 | 72 | 51.9 | 5 |
| *A.pullulans* HP-2001 | Soybean pomace | 5.7 | 96 | 7.5 | 6 |
| *A pullulans* NRRL Y-6220 | Soya Bean Oil | 4.0 | 120 | 17.4 | 7 |
| *A pullulans* NRRL Y-2311-1 | Soya Bean Oil | 3.5 | 96 | 26.24 | |
| *A.pullulans* ATCC 42023 | CSL+ sucrose | 2.2 | 120 | 65.3 | 8 |
| | clarified cane molasses | 2.2 | 120 | 47.84 | |
| | potato starchy waste | 3.5 | 120 | 22.33 | |
| | Hydrolysed sweet whey | 2.3 | 120 | 12.4 | |
| | hydrolysed rice straw | 4.0 | 120 | 9.36 | |
| *A.pullulans* MTCC 2195 | Cashew juice | 6.5 | 156 | 92.5 | 9 |
| | Maize | 6.5 | 96 | 71.0 | |
| | Cassava | 6.5 | 96 | 65.0 | |
| | Bakery waste | 6.5 | 96 | 27.0 | |
| *A.pullulans* AP329 | Sweet potato | 5.5 | 96 | 29.43 | 11 |
| *A.pullulans* MTCC 2195 | Coconut water | 7.0 | 144 | 38.3 | 10 |
| | Coconut milk | 7.0 | 144 | 58.0 | |
| *A. pullulans* MTCC 2195 | Jack fruit seed powder | 7.0 | 168 | 22.49 | 12 |
| *A.pullulans* RBF 4A3 | Jaggery+ DOJSC+ CSL | - | 72 | 66.25 | 13 |

CSL- Corn Steep Liquor, DOJSC- De-Oiled Jatropha Seed Cake

**Table 2.** Comparison of Pullulan production using Jaggery and Sucrose as carbon sources

| Carbon substrate | | Kinetic parameters | | | | | |
|---|---|---|---|---|---|---|---|
| **Type** | **Initial concentration, g/L** | **Pullulan content, g/L** | **Dry cell weight, g/L** | **Residual sugar, g/L** | **Yield, g Pullulan 100g sugar utilized** | **Pullulan/ productivity, g/(L.h)** | **Reference** |
| Jaggery | 50 | 10.8 | 45.2 | 3.2 | 21.6 | 0.062791 | This study |
| | 75 | 14.8 | 48.35 | 5.7 | 19.7 | 0.086047 | |
| | 100 | 18.6 | 73.01 | 14.3 | 18.6 | 0.10814 | |
| Sucrose | 50 | 9.7 | 39.0 | 3.2 | 19.4 | 0.056395 | |
| | 75 | 14.5 | 65.9 | 9.4 | 19.3 | 0.084302 | |
| | 100 | 17.5 | 60.1 | 14.3 | 17.5 | 0.101744 | |
| Jaggery | 50 | 23.01 | 7.25 | 5.68 | 51.9 | 0.239688 | 5 |
| | 75 | 24.56 | 9.24 | 11.25 | 44.61 | 0.255833 | |
| | 100 | 25.03 | 11.25 | 39.17 | 41.16 | 0.260729 | |
| Jaggery[a,] | 180 | 66.25 | — | — | 36.81 | 0.92014 | 13 |

[a,] - combination of Jaggery, De-oiled Jatropha Seed Cake, Corn Steep Liquor

elucidate what affects the fate of pullulan formation under different fermentation conditions, the fermentation have to be studied in5L reactor.

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