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Wanying Yao
University of Kentucky

Sue E. Nokes
University of Kentucky, sue.nokes@uky.edu

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Phanerochaete chrysosporium pretreatment of biomass to enhance solvent production in subsequent bacterial solid-substrate cultivation

Wanying Yao[1], Sue E Nokes[1*]

[1] Department of Biosystems and Agricultural Engineering, University of Kentucky

C.E. Barnhart Building, Lexington, KY, 40546

Phone: 859.257.3000 ext.128

Fax: 859.257.5671

Email: sue.nokes@uky.edu

Abstract: This study investigated pretreatment of corn stover using solid substrate cultivation (SSC) of *Phanerochaete chrysosporium (P. chrysosporium)* to improve subsequent accessibility to cellulose. Thereafter, *Clostridium thermocellum* (*C. thermocellum)* was directly inoculated onto the pretreated biomass to accomplish hydrolysis, followed by solventogenesis by introducing *Clostridium beijerinckii (C. beijerinckii).*

An enzyme suite containing laccase, lignin peroxidase and manganese peroxidase activity was detected during the cultivation of *P. chrysosporium* on corn stover within 288 h at an initial moisture content w_{H_2O} = 80 % (mass of water/total mass). Incubation factors, such as substrate moisture content and cultivation temperature affected the percent of lignin removal which ranged from 14.4 % to 36.4 % of the original lignin. Lignin removal increased as the cultivation of *P. chrysosporium* continued but was accompanied by increased cellulose loss. The 7-day fungal cultivation sufficiently delignified the corn stover for the subsequent processing. Approximately 25 % of the original lignin was removed; however 18 % of the initial cellulose was also removed with the lignin. The investigations of the effect of fungal pretreatment were extended to miscanthus, wheat straw and switch grass. The yield of reducing sugar produced by *C. thermocellum* on pretreated biomass was doubled compared with non-pretreated biomass, demonstrating that pretreatment resulted in a more accessible carbon source for the solvent-producing bacterium. The final comprehensive comparison between the pretreated biomass and non-pretreated biomass on the three-stage SSC for butanol production showed pretreatment by *P. chrysosporium* improved microbial utilization of lignocellulosic materials for solvent production by approximate 4 - 7 folds.

Key words: *P. chrysosporium*, lignin removal, cellulose hydrolysis *Clostridium thermocellum*, bacterial co-culture

1. Introduction

The global energy crisis has spurred interest in producing alternative biofuels from clean, renewable feedstocks via biological processes. Because of several desirable physical and chemical properties, butanol is currently a favored alternative to ethanol [1]. Butanol can be produced by an acetone, butanol and ethanol (ABE) fermentation via the anaerobic conversion of carbohydrates by *Clostridium* strains [2]. ABE fermentation was popular in the early 20th century. However, the ABE process eventually suffered from the high cost of conventional starch (maize, wheat, millet, etc.) or sugar (molasses) substrates, and was abandoned in favor of chemical synthesis of these chemicals. Current interest in biofuel production has stimulated research into the use of less expensive substrates [3, 4] for the ABE fermentation. Being the most abundant renewable resource, lignocellulose is recognized as a promising feedstock for use in biofuel fermentation, provided that the cellulose components can be deconstructed and utilized efficiently [5]. Since the ability of butanol-producing bacteria to catalyze the complex lignocellulosic feedstocks is limited, lignin removal or modification followed by cellulose hydrolysis is required to convert the lignocellulose into simpler sugars prior to the butanol fermentation. Chemical pretreatment combined with enzymatic saccharification of agricultural residues has been adopted as the conventional method to generate reducing sugars prior to solvent production [6-8]. However, inhibitors present after pretreatment and the high cost of hydrolytic enzymes have hindered process industrialization [9, 10]. These concerns motivated this study to develop a mild pretreatment with cost-effective hydrolysis for the ABE fermentation process.

Among the methods of pretreatment available, biological pretreatment with lignolytic enzymes is said to be superior to the current chemical and thermochemical methods in terms of energy intensity, environmental impact, and reduced production of chemicals toxic to fermentation microorganisms [11]. The enzymes commonly found in a lignolytic enzyme complex consist mainly of enzymes with lignin-degrading peroxidase activity (LiP; E.C.1.11.1.14), manganese peroxidase activity (MnP; E.C.1.11.1.13) and the lignin-degrading enzyme laccase activity (E.C.1.10.3.2). Some or all of these enzymes and their isozymes are produced by a number of wood-rotting fungi [12, 13]. The white-rot basidiomycete, *Phanerochaete chrysosporium (P.*

chrysosporium) is reported to have high lignolytic activity. This strain is considered to be a model strain for the development and understanding of the lignolytic enzyme production system because it can produce a more complete lignolytic enzyme complex than most other strains [13, 14].

Following pretreatment, the cellulose hydrolysis step could be accomplished using commercial enzymes (predominately fungal enzymes), however an alternative approach was used in this study. The alternative approach involved cultivating the solvent-producing *Clostridia* with a microorganism that can enzymatically convert cellulose into reducing sugars. There have been several reports of co-cultivations [15-17] using a cellulolytic strain like *Clostridium thermocellum* (*C. thermocellum), Clostridium cellulolyticum* or *Bacillus thermoamylovorans* to produce cellulase for saccharification of lignocellulose or cellulose (rice straw with swine dung, cellulose or solka floc), followed by butanol production achieved by adding a solventogenic species such as *Clostridium acetobutylicum* or *C. beijerinckii*. These studies report that sequential co-culture increased the total fermentation products formed from cellulosic substrate by 1.7 to 2.6-fold compared to *C. beijerinckii* monoculture.

The hypothesis for the current study was that a biological pretreatment followed by a sequential co-culture could efficiently utilize lignocellulosic substrate to produce solvents. To our knowledge, there are no previous studies investigating the effect of biological pretreatment on increasing substrate utilization and butanol yield, particularly for this co-culture process. Moreover, although sequential co-cultures have been investigated as a way of increasing the production of butanol using ideal feedstocks (pretreatment unnecessary), the studies were only performed in liquid fermentation. Solid substrate co-culture cultivation, which has been shown to have advantages over liquid cultivation [15] , has not been previously conducted using sequential co-cultures for ABE production.

To address this research gap, the objective of this study was to develop an appropriate biological pretreatment method which would increase the fermentability of lignocellulosic feedstocks intended for subsequent co-culture; specifically to increase cellulose accessibility and solvent accumulation in solid substrate cultivation. The fungal pretreatment of corn stover using SSC of

P. chrysosporium was investigated as a method to improve accessibility to cellulose in the pretreatment stage by preferentially degrading the lignin. Thereafter, *C. thermocellum* was directly inoculated onto the pretreated biomass to accomplish hydrolysis, followed by solvent production initiated by introducing *C. beijerinckii.* The study investigated the effect of cultivation conditions (moisture content of initial substrate and culture temperature) on the lignin removal by *P. chrysosporium* and then investigated the effects of lignin degradation on the subsequent fermentability by a bacterial co-culture, quantified by availability of carbohydrates and solvent production. A comprehensive analysis between fungal pretreated and non-pretreated biomass (corn stover, miscanthus, switch grass and wheat straw) on metabolite accumulation was performed to evaluate the effect of pretreatment on the solid substrate co-culture fermentation.

2. Materials and Methods

2.1 Strain cultivation

The white-rot basidiomycete, *P. chrysosporium* strain (ATCC MYA-4764) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained as a frozen culture (−80 °C) in 30 % glycerol. Propagation of the organism for SSC was performed as described by Shi et al. [16]. Spore suspensions were prepared by washing the slant with 10 cm^3 of sterilized sodium acetate buffer (50 mmol dm^{-3}, pH 4.5). The final spore inoculum concentration was 5×10^6 spores cm^{-3}, determined using a hemocytometer.

C. thermocellum ATCC 27405 was obtained from ATCC and grown in basal medium that contained (per L) [17]: 1530 mg Na_2HPO_4, 1500 mg KH_2PO_4, 500 mg NH_4Cl, 500 mg $(NH_4)_2SO_4$, 90 mg $MgCl_2 \cdot 6H_2O$, 30 mg $CaCl_2$, 4000 mg yeast extract, 10 cm^3 standard vitamins [18], 5 cm^3 modified metals [18], 500 mg cysteine hydrochloride, 1 cm^3 resazurin, and 4000 mg sodium carbonate. The medium pH was adjusted to 6.7 with NaOH and maintained under a 100% carbon dioxide atmosphere by sparging with CO_2 followed by sealing the container. *C. beijerinckii* ATCC 51743 purchased from ATCC was used in this study. For seed culture preparation, stock cultures were heat-shocked at 80 °C for 10 min, and transferred anaerobically into Reinforced Clostridial medium (RCM, Difco Laboratories, Detroit, Mich.) at 35 °C for 24 h.

2.2 Lignin analysis and biomass composition

The four types of biomass (corn stover, miscanthus, switch grass and wheat straw) used in this study were collected by rotary disc mower at 10 cm above the ground from the University of Kentucky North Farm in 2011. Switch grass (Alamo) and miscanthus (miscanthus x giganteus) were from the geographical coordinates 38.132396,-84.495948. Geographical coordinates for the wheat straw collection area were 38.080392,-84.737899. Corn stover was collected from the geographical coordinates 36.801519,-86.769254. Biomass samples consisted of the whole plant above ground (including stems and leaves), however grain had been previously removed from the corn stover and wheat straw. The biomass was stored in small square bales with $w_{H2O} < 15\%$ for 9 months. The bales were ground to 5 mm, thoroughly mixing the plant tissue. Samples were air-dried and stored at room temperature.

Biomass composition was determined using NREL methods [19]. Acid soluble lignin was measured using UV-vis spectrophotometry by quantifying absorption at 205 nm. The corn stover used in this study initially contained 21. 52 % of total lignin (Table 1) which consisted of 19.26 % acid insoluble and 2.25 % of acid soluble lignin. The analysis of lignin degradation (LD) was conducted following Shi et al. [20] as shown Eq. (1)

$$LD = 1 - \frac{\omega.(\alpha + \beta)}{\omega_0.(\alpha_0 + \beta_0)} \times 100\% \qquad \text{Equation 1}$$

Where ω is the dry weight of the treated sample (g); ω_0 is the initial dry weight of the untreated sample (g); α, β are the % of acid-soluble and acid-insoluble lignin respectively of the treated sample and α_0, β_0 are the % of acid-soluble and acid-insoluble lignin of the untreated sample.

2.3 Solid-substrate culture (SSC)

In SSC, the pretreatment of 0.5 g biomass by *P. chrysosporium* (50 mm^3 of spore culture) was conducted in 18 x 150mm modified Hungate tubes at 35 ^oC using cotton plugs in the tubes to allow for oxygen exchange with the environment. Prior to the inoculation of *C. thermocellum*, the tube with fungal pretreated biomass was flushed with CO_2 for 20 minutes and then sealed with a rubber topper and an aluminum seal finish. 2.0 cm^3 of standard inoculum of *C. thermocellum* was injected into the sealed test tube through the rubber stopper with a syringe. The CO_2 along with the gas generated from *C. thermocellum* maintain the anaerobic condition for the mono and co-culture. After a 2-day cultivation of *C. thermocellum* at 65 ^oC, an inoculum of 1 cm^3 *C. beijerinckii* at exponential phase per gram biomass was injected into the test tube through the rubber stopper. The co-culture was incubated at 35 ^oC in the water bath for 2 days. Destructive sampling of SSC bottles involved addition of 10 cm^3 100 mmol dm^{-3} potassium phosphate (pH 7) followed by centrifugation (5000 ×*g*, 10 min) of this mixture. The cell-free supernatant was stored at −20 °C for further analysis.

2.4. Measurement of enzyme activity from *P. chrysosporium*

Lignin peroxidase (LiP) was assayed on the basis of increased absorbance at 370 nm reflecting the oxidation change of veratryl alcohol to veratraldehyde [21]. One unit of the enzyme activity (1U) of LiP was defined as the amount of enzyme required to produce 1 μmol veratraldehyde per minute. The activity of MnP was determined by the increased absorbance at 240 nm corresponding to the oxidation change of Mn^{2+} to Mn^{3+} [22]. One unit of the enzyme activity of MnP was defined as 0.1 changes in absorbance per minute. The corresponding protocols can be found in Xu et al. [23]. The activity of laccase was determined by recording the increase of absorbance at 530 nm in a 3.00 cm^3 reaction mix containing 73 mmol dm^{-3} potassium phosphate, 0.02 mM syringaldazine, 10 % methanol, and 12.5 to 25.0 units laccase [24].

2.5 Analysis of co-culture's metabolite production

Fermentation samples (see section 2.3) were centrifuged at 9,600 × g and the products in the supernatants (lactic acid, acetic acid, butyric acid, ethanol, butanol and acetone) were quantified by high-performance liquid chromatography (HPLC) using a Dionex UltiMate 3000 system

(Dionex Corporation, Sunnyvale, California). A 300x7.8mm Aminex 87H column was maintained at 20 ^{o}C with a micro-guard cationic H cartridge at room temperature. The eluent was 5 mmol dm^{-3} H_2SO_4 at a flow rate 0.4 cm^3 min^{-1}. The metabolites were detected using shodex 101 refractive index detector.

2.6 Experimental Design and Data analysis

Experiments were conducted in two stages. First, the optimal moisture content was determined. SSCs were incubated at 35 ^{o}C for 10 days at 4 levels of w_{H_2O} = 50, 68, 80, 85 %. Next, the optimal moisture content was used to determine optimal culture temperature. SSCs were incubated for 10 days at w_{H_2O} of 80 % at 3 levels of temperatures (25, 30, 35 ^{o}C). All experiments were conducted in replicate and the data presented were mean values. ANOVA was employed with SPSS (Chicago, IL) to evaluate statistical differences between treatments. Statistical significance was defined as $p < 0.05$.

3. Results and Discussion

3.1 Enzymes suite and growth of *P. chrysosporium* on corn stover using SSC

Laccase, LiP and MnP activity was detected during the cultivation of *P. chrysosporium* (w_{H_2O} = 80 % by 288 h. Laccase activity was detected earlier than the activity of the other two enzymes (LiP and MnP) and reached the maximum (1.87 U cm^{-3} or about 49 U g^{-1} dry mass) after 166 h of cultivation but declined to about 1.20 U cm^{-3} in the following 60 h of cultivation. Genomic sequence searches of *P. chrysosporium* MYA revealed no conventional laccase sequence but did detect several multicopper oxidase sequences [25]. The detected laccase activity in our study may be attributed to these multi-copper oxidases. Because the conventional laccase is also a multi-copper oxidase and these related non-conventional oxidases may perform the extracellular oxidations. *P. chrysosporium* is known to decompose lignin by excreting extra-cellular peroxidases (LiP and MnP) under nutrient starvation [20, 26]. The production of LiP and MnP activity reached a maximum after 288 h at 0.248 U cm^{-3} (6.5 U g^{-1} dry mass) and 0.151 U cm^{-3} (4.0 U g^{-1} dry mass), respectively. The activity of all three enzymes stabilized around 288 hours and remained constant for the remaining of the experiment. Previous studies report that nitrogen

stimulated fungal growth and enzyme synthesis/activity for several white rot fungal strains in SmF [27, 28]. For SSC, Asgher [29] detected a maximum LiP activity of 7.26 U g^{-1} dry mass after 7 days SSC using corn cobs moistened with Kirk's basal medium. Further addition of yeast extract 0.2 % or peptone 0.3 % increased LiP activity to 11.27 U g^{-1} dry mass or to 12.26 U g^{-1} dry mass, respectively. In our study *P. chrysosporium* produced an enzyme suite of laccase, LiP and MnP activity with no nitrogen or carbon supplementation except what the fungus could extract from the corn stover substrate.

The composition and relative yields of the fungal enzyme activity have been reported to be strongly affected by the substrate type. Couto et al. [30] and Moldes et al. [31] reported the stimulating effect of lignin for laccase production by the white-rot fungus *Trametes versicolor* using lignocellulosic substrate. Gassara (2010) claimed that the high levels of insoluble carbohydrates, such as cellulose, hemicellulose, and lignin content of apple pomace stimulated high values of laccase and peroxidase (LiP and MnP) synthesis/activity by *P. chrysosporium*. Our results differs from previous reports [32-34] in the relative composition of enzyme suite, which was expected because we used a different substrate. In addition, we saw a strong correlation between mycelial growth and peroxidase (LiP and MnP) activity. The timing of the initial measurement of LiP and MnP activity is consistent with the increase in fungal mycelia growth visually observed after 5 days culture (Figure 2).

Figure 2 presents the mycelial growth of *P. chrysosporium* during the 11–day SSC. SSC processes differ from SmF, since microbial growth initiates at the surface of the solid substrate particles while mycelial pellets are typically formed in SmF [35]. In the current study, the mycelia of *P. chrysosporium* initiated on the surface of the corn stover presumably because oxygen could be easily accessed. Accumulated mycelia were observable on the surface of the corn stover after 5 days. As the cultivation continued, the mycelia gradually penetrated deeper into the biomass and finally through the entire biomass after 11 days of culture. No substantial mycelial growth was observed after day 11. The conventional opinion states that the success of the SSC is directly related to the appropriate particle size of the support, which influences both nutrient diffusion and fungal cell attachment [36]. Generally, smaller substrate particles provide a larger surface area for microbial colonization but particles which are too small may result in poor growth due to substrate agglomeration which restricts nutrient and oxygen accessibility. In

contrast, larger particles provide better aeration but a limited surface for microbial colonization [37]. A particle size of 5 mm seemed to be an appropriate size to support microbial activity as evidenced above.

3.2 Effect of cultivation conditions on lignin removal of corn stover

The percent of lignin removed is positively correlated with the water content of initial substrate as seen in Fig.3. Water in SSC functions as a solvent to transport nutrients as well as to help maintain stable cellular and molecular structures [20, 38]. Although fungal growth can be initiated at w_{H_2O} of 50 %, the metabolic functions of *P. chrysosporium* apparently cannot be actively supported, as evidenced by only 15 % of the original lignin in corn stover removed. The w_{H_2O} = 80 % and w_{H_2O} = 85 % treatments demonstrated approximately 35 % lignin degradation after 10 days. Our results demonstrated that moisture content affected fungal growth as well as the lignin degrading performance in SSC systems, which was also reported in As claimed by Lousane et al. [39] and Mitchell et al. [40]. These results were also consistent with the observation that *P. chrysosporium* in SSC requires moisture contents ranging between 40 % and 90 % [29]. Ironically, higher moisture contents limit oxygen transfer, often inhibiting aerobic SSC cultures, and increasing susceptibility to bacterial contamination [20, 29], however we did not see this effect in our cultures.

Cultivation temperature had a significant effect on lignin removal (Figure 4). Up to 35 % acid insoluble lignin and 21 % acid soluble lignin of the corn stover was removed when cultivated at 30 ^{o}C to 35 ^{o}C, which was significantly higher than the lignin removed at 25^{o}C (approximately 15 % ASL). No effects of temperature were observed for acid insoluble lignin removal.

3.3 Effect of fungal pretreatment on *C. thermocellum* growth and metabolites

Ideally an effective fungal pretreatment would reduce the percentage of lignin in the original substrate while simultaneously conserving the cellulose polymers. In our study, a direct relationship between cellulose loss and lignin removal was observed in corn stover. An increase in lignin removal from 14. 37 % to 36.40 % was accompanied by an increasing loss of cellulose from 11.78 % to 28.75 %, likely because more cellulose was accessible to the fungus as its carbon source through decomposition of the lignin structure. An approximate cellulolytic activity of 0.1 U cm^{-3} (2.14 U g^{-1} initial substrate) was detected in the washed broth of the 11th day SSC. The cellulase secreted by *P. chrysosporium* facilitated the enzymatic degradation of cellulose to sugars available for use by the fungi, which lowered the overall availability of carbohydrates for the subsequent fermentation. Others have observed cellulolytic activity in wood-rotting fungi [41]. Once total lignin content dropped below 14.2 % of the total biomass composition, no additional improvement in metabolite accumulation by *C. thermocellum* was observed. Ethanol was also produced during the fungal fermentation. The ethanol content ranged from 0.10 to 0.85 mg g^{-1} corn stover in treatments where the lignin removal was 14.4 - 36.4 % of the total lignin respectively. Some researchers also claim that several species of white-rot fungi are capable of producing ethanol (0.17 - 0.42 g g^{-1} substrate) directly from cellulosic biomass [42, 43]. However, *P. chrysosporium* is not a major ethanol producer. The major role of *P. chrysosporium* utilized in this study was to delignify the biomass for subsequent processing steps, and ethanol was not the focus of our study.

Typically, ethanol and acetate are the main extracellular metabolites produced by C. *thermocellum* monocultures. In our study, increasing the percent lignin removal from 14.4 % to 27.3 % had a positive effect on glucose and cellobiose generated by *C. thermocellum*'s enzyme system (but not total metabolites produced – see above) with the largest amount being 19.17 mg glucose g^{-1} initial substrate and 18.12 mg cellobiose g^{-1} initial substrate, respectively but lignin removal had a limited effect on acetate production. Further lignin removal from 27.3 % to 36.4 % did not increase metabolite (ethanol and acetate) accumulation however the cultures did consume more of the carbon source (glucose and cellobiose) ideally meant for the subsequent fermentation. This can be seen in Table 2, where an increase in lignin removal from 27. 3 % to

36.4 % resulted in an increased loss of cellulose from 18.78 % to 30.75 %. Our data inferred that the optimal pretreatment with 25 - 27 % of lignin removed (7-day cultivation) saved more cellulose for the subsequent cultivation.

3.4 Evaluation of *P. chrysosporium* pretreatment method on solid substrate batch sequential co-culture of *C. thermocellum* and *C. beijerinckii* on four types of biomass

The yield of glucose produced by *C. thermocellum* on biomass pretreated with *P. chrysosporium* was at least twice that of non-pretreated biomass (Fig. 5) for all four types of biomass, corn stover, miscanthus, switchgrass. We hypothesize that lignin removal by *P. chrysosporium* exposed the cellulose to the bacterial enzyme systems, leading to the efficient access and utilization of cellulose resulting in more accessible carbon available for the solvent phase microorganism.

All treatments produced approximately the same amount of ethanol (Fig. 5). However, becasue the co-cultivation for solvent production is the ultimate goal of our project, significant metabolite production during the *C. thermocellum* phase was not a priority, and in fact was undersirable. Ideally the *C. thermocellum* step in the process would leave only carbohydrates for the sequential co-culture to use as feedstock for *C. beijerinckii* to produce acids and solvents. The acids produced by *C. thermocellum* should not exceed a critical point to avoid creating unfavorable osmotic conditions for *C. beijerinckii*, resulting in metabolic inhibition [17]. Thus it is essential to not only adopt an effective pretreatment method with little carbon loss or inhibitors produced but also control the metabolite production of *C. thermocellum* in order to direct the cultivation stage transition and sustain an appropriate environment for *C. beijerinckii* in the sequential co-culture. In summary, the fungal pretreated biomass showed better performance than non-pretreated ones in terms of cellulose hydrolysis by *C. thermocellum* and in retaining carbon for *C. beijerinckii*. Significantly more butanol was produced from the fungal pretreated biomass as compared to non-pretreated biomass (Table 3) for all types of biomass investigated. The fungal-pretreated miscanthus corresponded to the highest yield of butanol, being 3.66 mg g^{-1} biomass (equal to a titre of 0.6 g dm^{-3}). The study confirmed the positive effect on lignin removal by fungal pretreatment when using corn stover in a sequential co-culture ABE fermentation and extended the established three-stage SSC model to wheat straw, switch grass, and miscanthus.

1. Conclusion

The study investigated the effect of solid substrate cultivation conditions on the lignin removal by *P. chrysosporium*, and the subsequent fermentability of the pretreated substrate by a bacterial co-culture. Up to 36.4 % of total lignin was removed when cultivation of *P. chrysosporium* was maintained at 35 ^{o}C and w_{H_2O} = 80 - 85 % but accompanied by the cellulose consumed as well. The optimal pretreatment resulted in a 25 - 27 % of lignin removed (7-day cultivation of *P. chrysosporium*), which saved more cellulose for the sequential co-cultivation. *C. thermocellum* in SSC grown on fungal pretreated biomass degraded the substrate into a significantly higher amount of glucose than cultures grown on non-pretreated biomass. Significantly higher yields of butanol were generated by fermenting fungal pretreated biomass compared to the non-pretreated biomass. Those observations demonstrated the efficient conversion of lignocellulosic material after fungal pretreatment, which resulted in more value-added acids and solvents.

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