การคัดแยกและคัดกรองยีสต์สะสมไขมันสูงที่สามารถใช้กลีเซอรอลเป็นแหล่งคาร์บอน

Isolation and Screening of Oleaginous Yeasts Capable of Using Glycerol as a Carbon Source

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บทคัดย่อ

ยีสต์สะสมไขมันสูงเป็นผู้ผลิตไขมันที่มีศักยภาพสำหรับนำมาใช้ประโยชน์สำหรับการผลิตไบโอดีเซล การศึกษาครั้งนี้จึงได้ทำการเก็บตัวอย่างจากใบไม้และดินจากบริเวณป่าชายเลน จังหวัดชลบุรี แล้วนำมาคัดแยกยีสต์โดยใช้อาหารเลี้ยงเชื้อ YEPG broth ที่มีกลีเซอรอล 2 เปอร์เซ็นต์เป็นแหล่งคาร์บอนในขวดรูปชมพู่ภายใต้สภาวะแบบเขย่า พบว่าสามารถคัดแยกยีสต์ได้ทั้งหมด 26 ไอโซเลท จากนั้นนำยีสต์ที่คัดแยกได้มาวิเคราะห์ความสามารถในการสะสมไขมันโดยการย้อมด้วยสี Sudan Black B พบว่าจากยีสต์ทั้งหมด 26 ไอโซเลทที่คัดแยกได้ มียีสต์ 7 ไอโซเลท ได้แก่ 1A 2C 3E 5B 6A 8A และ 9A ที่มีถุงสะสมไขมันภายในเซลล์ จึงนำยีสต์ทั้ง 7 ไอโซเลทมาประเมินปริมาณไขมันภายในเซลล์ด้วยวิธี Nile Red fluorescence assay พบว่ายีสต์ไอโซเลท 1A ที่คัดแยกได้จากใบของต้นแสมดำแสดงศักยภาพในการสะสมไขมันสูงที่สุด เมื่อนำไอโซเลท 1A ไปศึกษาจลศาสตร์ของการผลิตชีวมวลและไขมัน พบว่าที่เวลา 240 ชั่วโมงของการเพาะเลี้ยงในอาหาร GMY broth ไอโซเลท 1A ผลิตชีวมวลได้ 24.24 ± 0.06 กรัมต่อลิตร สะสมไขมันได้ 3.63 ± 0.04 กรัมต่อลิตร คิดเป็นปริมาณไขมันสูงสุด 14.98 เปอร์เซ็นต์ของชีวมวลแห้งอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) อย่างไรก็ตาม ควรที่จะต้องศึกษาเพิ่มเติมเพื่อปรับปรุงการผลิตไขมันของยีสต์ไอโซเลท 1A ก่อนที่จะนำไปประยุกต์ใช้ในอนาคต

คำสำคัญ : กลีเซอรอล ลิพิดจากจุลินทรีย์ ยีสต์สะสมไขมันสูง

ABSTRACT

Oleaginous yeasts are potential lipid producers that could be used for biodiesel production. In this present study, leaf and soil samples from a mangrove forest in Chonburi Province were collected and used for the isolation of yeasts in YEPG broth containing 2% glycerol as a carbon source in shaking flasks. The total of 26 isolates of yeast were obtained and they were further investigated for their ability to accumulate lipid by Sudan Black B staining. The result showed that 7 out of 26 yeast isolates; 1A 2C 3E 5B 6A 8A and 9A had intracellular lipid droplets. All of these isolates were estimated for their approximate amount of lipid accumulation by Nile Red fluorescence assay and only one isolate namely 1A that was isolated from leaves of *Avicennia officinalis* expressed the strongest potential for high level of lipid accumulation. After kinetic studies of biomass and lipid production of isolate 1A at 240 hours of growth in GMY broth, 24.24

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± 0.06 g/L of biomass yield, 3.63 ± 0.04 g/L of lipid yield, and lipid content of 14.98% of dry biomass were significantly achieved ($p < 0.05$). Strategies for improvement on lipid production from this isolate is needed to be further investigated prior to its utilization in the future.

Keywords: glycerol, microbial lipid, oleaginous yeast

Introduction

Recently, demands on biodiesel as a biofuel have been increasing in America, Europe, and Asia since it is considered as an environmental-friendly alternative fuel (Eamcharoen and Rungrojchaipon, 2016). Glycerol is an industrial waste derived from biodiesel production via transesterification or alcoholysis of vegetable oils. Generally, proportion of 10% (w/w) of crude glycerol is produced from biodiesel (Santibañez et al., 2011). Yeasts could convert glycerol to intermediate compounds and eventually produce dihydroxy-acetone phosphate as a product via glycolytic pathway. Dihydroxyacetone phosphate is further changed to citric acid which is subsequently converted to storage lipid (Rakicka et al., 2015). Therefore, application of glycerol as a carbon source for the growth of oleaginous microorganisms such as oleaginous yeasts (Kitcha and Cheirsilp, 2011) would be attractive in terms of waste removal and cost reduction for microbial lipid production.

Oleaginous microorganisms are those such as yeasts, molds and microalgae that could accumulate lipid above 20% of their biomass (Evan and Ratledge, 1984). Lipid accumulation in yeasts usually takes place when carbon is in excess and nitrogen is limited (Amaretti et al., 2010). Some genera of oleaginous yeasts; *Rhodosporidium sp., Rhodotorula sp.*, and *Lipomyces sp.* could accumulate lipid up to 70% of dry biomass (Li et al., 2008). Oleaginous yeasts store intracellular lipid in lipid droplets that are distributed in the cytosol. Lipid content varies between different yeast strains as well as cultivation conditions (Cuimin et al., 2009). Major composition of yeast lipid is triacylglycerol (TAG). In addition, lipid of oleaginous yeasts has fatty acid profiles similarly to those of vegetable oils that are commonly used as substrates for biodiesel production via transesterification reaction (Sitepu et al., 2014). Thus, this is a great potential to replace vegetable oils for biodiesel production in the future. Taking that into an account, when yeast lipid is utilized as a substrate in transesterification, glycerol would also be produced alongside biodiesel. Subsequently, the obtained waste glycerol could be recycled by applying it as a carbon source for growth and lipid accumulation of oleaginous yeasts again. These processes could be carried out indefinitely to exploit the full potential of both yeast lipid and glycerol.

Oleaginous yeasts are widely distributed in natural habitats (Jiru et al., 2016), e.g. soils, leaves, flowers, and from various environments as well as mangrove forests (Kunthipun et al., 2018).

Therefore, the aim of this work is to isolate and screen for potential oleaginous yeasts capable of using glycerol as a carbon source from biomaterials at a local mangrove forest. Lipid content of a selected yeast isolate was also investigated.

Methods

Sample collection

Five samples of leaves from five different plant species (*Rhizophora mucronata* Poir., *Rhizophora apiculata* Blume, *Xylocarpus moluccensis* (Lamk) M.Roem., *Avicennia officinalis* L. and *Ceriops tagal* C.B.Rob.) and five soil samples near five different *Rhizophora apiculata* Blume trees were collected from Mangrove Forest Conserve and Natural Study Center in Chonburi province, Thailand. Leaves were cut from the living trees and placed in sterile plastic bags (One plant species per bag). Each soil sample was taken at the depth of 2 cm under the soil surface and collected in a sterile plastic bag. All samples were transported to the lab for the isolation of yeasts.

Isolation of yeast strains

After cutting into small pieces, 2 g of each leaf sample was transferred to a 250-ml Erlenmeyer flask containing 100 ml of YEPG broth (20 g/l peptone, 10 g/l yeast extract, 20 g/l pure glycerol) with the addition of 0.05 g/l chloramphenicol. Five grams of each soil sample was added to a 250-ml Erlenmeyer flask containing 100 ml of YEPG broth. All flasks were incubated at room temperature with 150 rpm for 3-5 days. Samples were diluted in 0.85% NaCl and spreaded on YEPG agar (YEPG broth with 2% agar and 0.05 g/l chloramphenicol). Colonies with different morphologies were picked, observed under a light microscope and streaked on freshly-prepared YEPG agar plates to obtain pure cultures.

Primary screening of oleaginous yeasts by Sudan Black B staining

A few colonies of isolated yeast strain were added to 100 ml of GMY broth containing 4% (w/v) pure glycerol and 0.3% (w/v) yeast extract as a carbon source and a nitrogen source, respectively (Munch et al., 2015), with initial $O.D._{600}$ of 0.02. Flasks were incubated at room temperature with 150 rpm. Five-day cultures were used for Sudan Black B staining. After smear preparation, the experiment was followed by air dry, heat fix, flooding the smear with Sudan Black B solution for 15 minutes, discarding the solution, and flooding the smear with 0.5% Safranin O solution for 30 seconds (Burdon, 1946; Jape et al., 2014). Presence of lipid droplets in the cytosol of yeast cells were observed under a light microscope.

Secondary screening of oleaginous yeasts by Nile Red fluorescence assay (Sitepu, et al., 2012)

Selected yeast isolates from the primary screening were cultivated in 100 ml of GMY broth with initial $O.D._{600}$ of 0.02 at room temperature with 150 rpm for 5 days. Yeast culture was further adjusted the $O.D._{600}$ to 1.00 for further use in Nile Red-containing mixture. A Nile Red-containing mixture for each yeast culture was prepared in triplicate. A Nile Red-containing mixture contained 250 µl of yeast culture, 25 µl of DMSO/GMY broth (1:1, v/v) and 5 µg/ml of Nile red. The 200 µl of the mixture was transferred to each well of a black microtiter plate and fluorescence intensity emitted from Nile Red stained lipid was measured in a fluorescence spectrophotometer using excitation and emission wavelength at 530 and 590 nm,respectively.

Kinetic studies of biomass and lipid production of a selected yeast strain

A selected yeast strain from secondary screening was transferred to 100 ml of GMY broth with initial $O.D._{600}$ of 0.02 and incubated at room temperature with 200 rpm for 10 days. Yeast cultures were collected every 12 hours for investigating the biomass and lipid production by measuring the weight of dry biomass after drying in a hot air oven at 60ºC until the weight was constant. The experiments were carried out in triplicate. Dry biomass was further used for lipid extraction (Tapia et al., 2012; Castanha et al., 2013) by treating with 2M HCl to disrupt cell wall with a proportion of 2M HCl : dry biomass as 4 ml : 0.3 g. After the mixture was incubated for 1 hour at room temperature, the acid was discarded and then cells were mixed with 4 ml of sterile distilled water, 10 ml of methanol and 5 ml of chloroform. After being incubated with shaking at room temperature for 2 hours, the mixture was centrifuged at 180 *x g* for 2 minutes. Lipid was obtained by collecting the chloroform layer and let the chloroform evaporated and dried at 60ºC. The obtained lipid was weighed and further calculated lipid content using the following formula;

Lipid content (%) = [Dry lipid weight (g/l) / dry cell weight (g/l)] * 100

Statistical analysis

Values of Nile Red fluorescence intensities, biomass production, lipid production and lipid content were analyzed independently for the statistical significance at p-value < 0.05 using one-way ANOVA in IBM SPSS Statistics (version 22). Data (n = 3) are presented as means ± standard deviation following by different lowercase alphabets which represent significantly difference between each value.

Results

Isolation of yeast strains

In this study, 26 yeast isolates were collected from leaf and soil samples from Mangrove Forest Conserve and Natural Study Center in Chonburi province. Most of yeast strains were isolated from five leaf samples (5 isolates from *Rhizophora mucronata* Poir., 2 isolates from *Rhizo-phora apiculata* Blume, 6 isolates from *Xylocarpus moluccensis* (Lamk) M.Roem., 4 isolates from *Avi-cennia officinalis* L. and 4 isolates from *Ceriops tagal* C.B.Rob.). Moreover, 5 yeast isolates were obtained from soils. Each isolate conferred different colony morphologies on YEPG agar, some of them are exhibited in Table 1. Additionally, cells of each isolate appeared similarly from ovoidal to spherical in shape and reproduced asexually by budding (data not shown) which were typical characteristics of yeast cells.

Primary screening of oleaginous yeasts by Sudan Black B staining

All of yeast isolates were grown for 5 days in GMY broth, which is a nitrogen-limited medium providing essential condition to induce high amount of lipid accumulation in oleaginous yeasts (Amaretti et al., 2010), and subjected to Sudan Black B staining to determine the potential of each yeast isolate for lipid accumulation. The results showed that, from a total of 26 yeast isolates tested, lipid accumulation was observed in 7 yeast isolates; 1A 2C 3E 5B 6A 8A and 9A. As shown in Table 2, grey to black and round-shaped intracellular lipid droplets in red cytosol of these isolates were observed, especially in isolates 1A and 2C which had evident lipid accumulation in terms of dye intensity and large lipid droplets.

Secondary screening of oleaginous yeasts by Nile Red fluorescence assay

Seven yeast isolates that showed lipid accumulation based on Sudan Black B results were further cultivated in GMY broth for five days and proceeded to Nile Red fluorescence assay. The results showed that isolate 1A had the highest level of fluorescence intensity (Figure 1) which implied that isolate 1A was able to accumulate the highest amount of lipid in comparison to other yeast strains tested.

Kinetic studies of biomass and lipid production of a selected yeast strain

Based on its highest potential in lipid accumulation, isolate 1A was selected for time course study of biomass and lipid production in GMY broth. As shown in Figure 2, it was found that lipid production of isolate 1A was relatively correlated with biomass production. The maximum biomass production, lipid production and lipid content were significantly (p < 0.05) achieved 24.24 ± 0.06^{l} g/l, 3.63 ± 0.04^{e} g/l and $14.98\%^{k}$ of dry biomass, respectively, at the end of incubation (240 hours) which was during stationary phase of growth.

Table 1 Colony morphology of some yeast isolates obtained from leaf and soil samples.

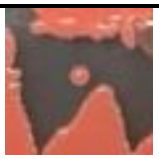
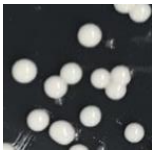
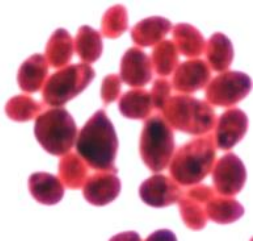
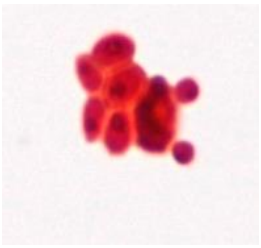
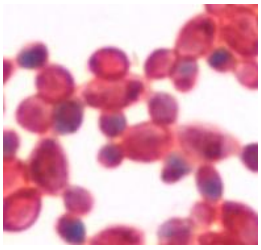
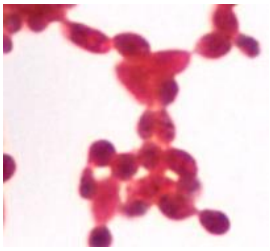
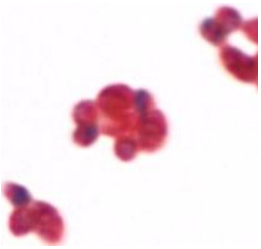
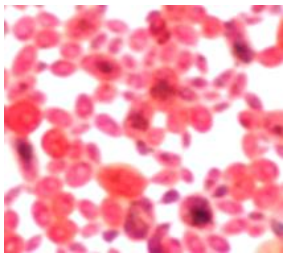
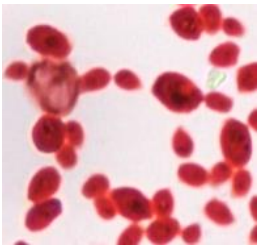
Yeast isolates	Colony morphology						Representative images
	Form	Elevation	Margin	Surface	Color	Diameter (mm)	
1A	Circular	Umbonate	Entire	Glistening	Pink	1	
2C	Filamentous	Flat	Filiform	Rough	Whitish cream	1	
3A	Circular	Convex	Undulate	Glistening	Cream	1.90	
8A	Circular	Convex	Entire	Glistening	White	1.50	

Table 2 Lipid was stained with Sudan Black B in the cells of 7 yeast isolates. Images were captured with a total magnification of 1000x under a light microscope.

Yeast isolate	Source	Cells stained with Sudan Black B	Yeast isolate	Source	Cells stained with Sudan Black B
1A	*Avicennia officinalis* L.		6A	*Ceriops tagal* C.B.Rob.	
2C	*Rhizophora mucronata* Poir.		8A	*Avicennia officinalis* L.	
3E	*Xylocarpus moluccensis* (Lamk) M.Roem.		9A	Soil sample	
5B	*Rhizophora apiculata* Blume				

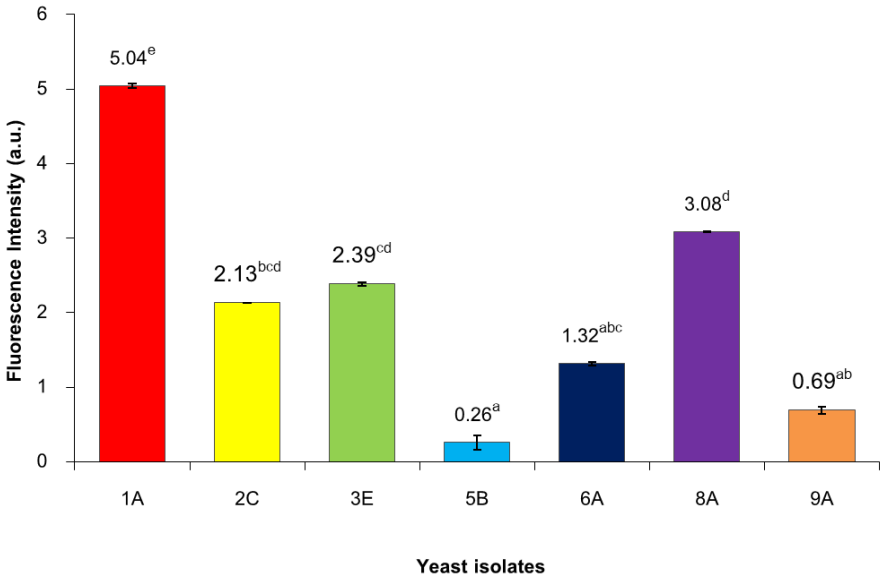


Figure 1 Fluorescence intensities of seven yeast isolates that were stained with Nile Red.(Different lowercase alphabets after each value represent significantly difference, $p < 0.05$)

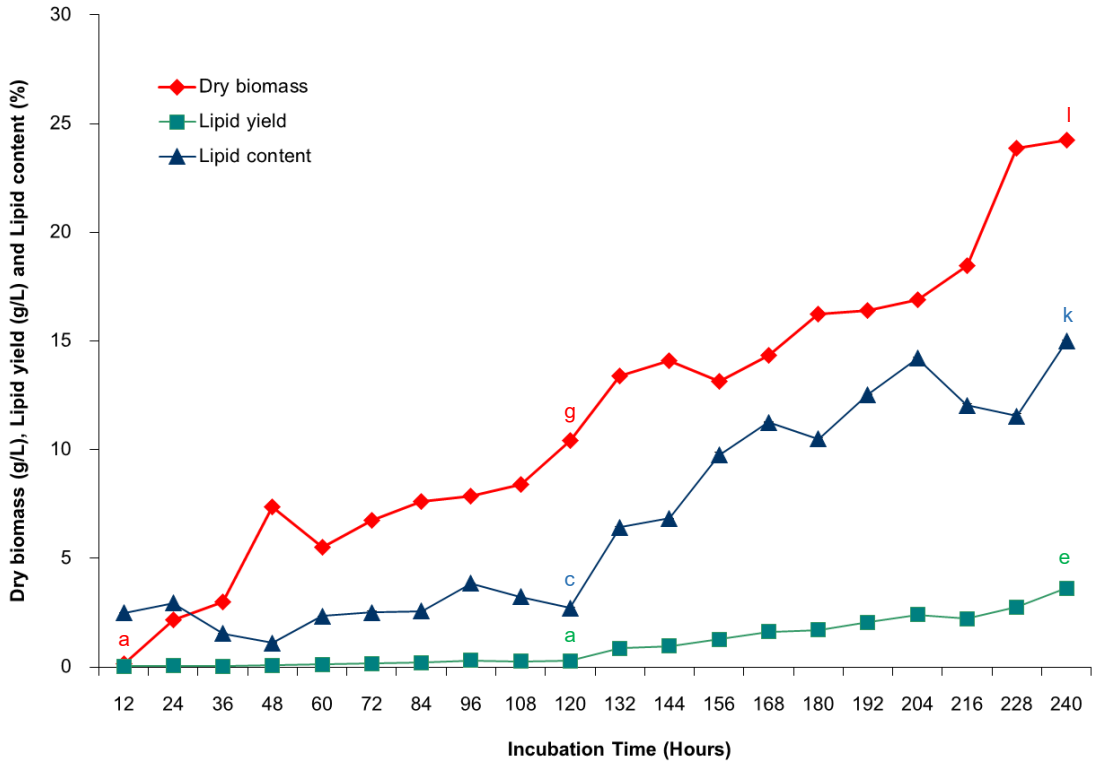


Figure 2 Biomass yield, lipid yield and lipid content achieved by isolate 1A cultivated in GMY broth for 10 days. (Different alphabets above time points represent significantly difference, $p < 0.05$)

Conclusion and Discussion

In this study, 21 yeast isolates could be isolated from five leaf samples and 5 yeast isolates from soils resided in mangrove forest. This result implied that leaves and soils are decent sources for the isolation of yeasts since they are common inhabitants of leaf and could be found in various soils (Jiru et al., 2016).

Further experiments showed that approximately 27% of the total yeast isolates, based on Sudan Black B staining, were oleaginous yeasts. Sudan Black B is a lipid-soluble dye capable of staining intracellular lipid (Jape et al., 2014). In this study, lipid droplets stained with Sudan Black B were observed in 7 yeast isolates which were in concordance with a previous published report that also observed lipid droplets in the following genera of yeasts; *Candida* spp., *Candida tropicalis* and *Rhodotorula mucilaginosa* using Sudan Black B staining (Jape et al., 2014).

Nile Red fluorescence assay was also utilized for the selection of an oleaginous yeast with the highest potential for lipid accumulation. Nile red is a fluorogenic dye that could stain lipid and its level of fluorescence intensity is dependent on the amount of lipid in the cells (Sitepu et al., 2012).

Results from Nile Red fluorescence assay were in concordance with results from Sudan Black B staining which implies that we may use either or both techniques to simply qualitatively screen for oleaginous yeasts. However, these techniques could not be used to directly quantify the concentration of lipid. Hence, based on yeast's ability to accumulate high lipid content, we found that strain 1A was the most promising strain according to the presence of prominent intracellular lipid stained by Sudan Black B and the highest level of Nile Red fluorescence intensity in comparison to other isolated oleaginous yeast strains.

The investigation of biomass and lipid production of isolate 1A for over 10 days in a nitrogen-limited medium revealed that the maximum lipid content (14.98%) of isolate 1A was higher than *Rhodotorula glutinis, R. mucilaginosa, R. rubra* and *Sporobolomyces salmonicolor* that had lipid contents of 9.1%, 10.2%, 7.5% and 4.2%, respectively, in a medium that contained pure glycerol (Gientka et al., 2017). This result is in contrast to other reports that showed higher level of lipid content. For example, Munch et al. (2015) who found that *Rhodosporidium babjevae* and *Rhodosporidium diobovatum* had lipid contents of 34.9±3.0 and 63.7±4.5% of dry biomass, respectively, in the presence of pure glycerol in a nitrogen-limited medium. Castrillón et al. (2017) found that *Meyerozyma guilliermondii* BI281A could accumulate lipid with 34.97% of dry biomass.

Those previous reports as well as this study demonstrated that lipid contents could be varied in different genera, species and strains of yeasts, culture media, types of carbon and nitrogen sources, C/N ratio, and cultivation time etc. In this study, GMY medium was used for yeast cultivation since it had C/N ratio around 50 which was adequate to stimulate lipid accumulation in yeasts (Papanikolaou and Aggelis, 2011).

Lipid content in isolate 1A could be enhanced by further optimization of the cultivation conditions. The residual concentration of carbon source and nitrogen source should be further measured along with biomass and lipid production in order to understand the correlation between lipid production and substrate consumption. Additionally, identification of yeast isolates should be carried out to obtain more information of yeast diversity in the mangrove forest.

In conclusion, our research showed that oleaginous yeasts could be obtained from biomaterials at a mangrove forest which is a natural and valuable resource for the search of new beneficial yeast strains. In addition, these oleaginous yeast isolates could use glycerol which is a promising carbon source for growth and lipid accumulation.

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