DEVELOPMENT OF LIPASE IMMOBILIZATION TECHNIQUE USING SAGO AS SUPPORT FOR ENZYMATIC ESTERIFICATION AND TRANSESTERIFICATION

By

NUR SYAZWANI BINTI MOHTAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

December 2020

IPPH 2021 8

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chair : Raja Noor Zaliha Raja Abd Rahman, PhD
Faculty : Halal Products Research Institute

One of the major enzymes produced and used in industries is lipase. Lipases are very versatile catalyst and its applications expand to various industries like oil and fat, dairy, pharmaceutical, detergent, leather, cosmetics and paper. The hitch is enzymes are very sensitive to the change in the environment like temperature and pH. One way to resolve this problem is to immobilize the desired enzyme on a support material. Enzyme immobilization does not only stabilize the enzyme, but it also makes the enzyme suitable to be used for reactions that are sensitive to water because the immobilized enzymes are often in a dry form. This is very important for lipase, as the majority use of lipase in the industry is for ester synthesis and fat modification (transesterification), which only occur in a water-limiting condition. Therefore, the objectives of this research are to develop a simple and effective method to immobilize lipase, using sago as the support, and test the immobilized lipase for esterification and transesterification activity. Sago is the starch extracted from the pith of *Metroxylon sagu* palm trees. It is deemed suitable as the immobilization support due to its inert nature, stable gel structure and excellent resistance to heat and shear.

A thermostable lipase isolated from *Geobacillus* sp. ARM (ARM lipase) was used in the immobilization study to develop a simple and efficient method to increase the stability of the enzyme. Pure ARM lipase was used to minimize the contaminant that might affect the results of the experiments. Two immobilization techniques using different supports were tested, that is, adsorption on chitosan, amberlite, and layered double hydroxide and entrapment in sago. The highest lipase activity determined was by entrapment of the lipase in gelatinized sago that subsequently spray-dried. The result was supported by the surface area and

porosity analysis where the surface area, pore volume, and pore radius decreased by 67%, 50%, and 10%, respectively, when lipase was immobilized into the sago, which indicated that the space had been occupied by the enzyme. This immobilization method has efficiently improved the thermal stability of the enzyme, where the half-life of the enzyme at 80°C was 4 hours, in comparison to the activity of the free enzyme, which dropped instantly from the very first hour. This immobilization method has also successfully preserved the enzyme for a longer shelf-life, which was 9 months at 10°C and 2 weeks at room temperature. The immobilized ARM lipase maintained its optimum temperature for enzyme activity at 70°C and a pH preference from pH7 to pH9. Overall, the results of thermal and pH characterization have shown that the immobilization of lipase on a natural support material such as sago is very promising for industrial use, especially in food manufacturing applications.

Two model applications were done to test the lipase immobilized using the newly developed method for synthesis activity in a water-free environment. The immobilized enzyme was used for the synthesis of ethyl oleate by esterification reaction and the synthesis of cocoa butter alternative by transesterification reaction. The usage of dry immobilized lipase is very relevant for these applications. For these experiments, a commercial enzyme, *Rhizopus oryzae* lipase (ROL), which was immobilized using the newly developed method was used to compare with the ARM lipase. The immobilized ROL showed an outstanding result for the esterification (81.3% at 60°C) compared to ARM lipase (9.9% at 60°C), therefore, it is used for further experiments. The highest conversion of ethyl oleate obtained using the immobilized ROL from this research was 97% with the incubation in a water bath shaker at 40°C and 50°C, 200 rpm shake, for 12 hours, with the substrate ratio of ethanol and oleic acid of 2:1. For the synthesis of cocoa butter alternative using the immobilized ROL in sago, different ratios of palm mid-fraction, olive oil, and stearic acid were used as the substrates. Based on the chemical composition analysis determined by the high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GCMS), the product using substrate ratio 1:0:1 has the closest reading to cocoa butter compared to the other ratios, with 21.77% palmitoyl-oleoyl-palmitoyl glycerol, 27.71% palmitoyl-oleoyl-stearoyl glycerol, 13.39% stearoyl-oleoyl-stearoyl glycerol, the total saturated fatty acids of 66.07%, and unsaturated fatty acids of 33.93%. As for the thermal characteristics, the product using substrate ratio 1:1:1 has the closest reading to cocoa butter compared to the other ratios, with the slip melting point of 36.2°C; the solid fat content of 7.73% and 2.02% at 30°C and 40°C respectively. The DSC thermogram showed melting peaks at lower than 37°C (11.56°C and 33.41°C). In a nutshell, the newly developed method of enzyme immobilization using sago as the supporting material is suitable for lipases from a bacterial and a fungal source, for the forward and reverse reactions of lipase.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN TEKNIK PENYEKAT-GERAKAN LIPASE DENGAN MENGGUNAKAN SAGU SEBAGAI BAHAN SOKONGAN BAGI ESTERIFIKASI DAN TRANSESTERIFIKASI BERENZIM

Oleh

NUR SYAZWANI BINTI MOHTAR

Disember 2020

Pengerusi : Raja Noor Zaliha Raja Abd Rahman, PhD
Fakulti : Institut Penyelidikan Produk Halal

Salah satu enzim utama yang dihasilkan dan digunakan dalam industri adalah lipase. Lipase adalah pemangkin serbaguna dan aplikasinya berkembang ke pelbagai industri seperti minyak dan lemak, tenusu, farmasi, detergen, kulit, kosmetik dan kertas. Masalahnya ialah enzim sangat sensitif terhadap perubahan persekitaran seperti suhu dan pH. Salah satu cara untuk mengatasi masalah ini adalah dengan menyekat-gerak enzim yang dikehendaki pada bahan sokongan. Penyekat-gerakan enzim tidak hanya menstabilkan enzim, bahkan juga menjadikan enzim tersebut sesuai digunakan untuk tindak balas yang sensitif terhadap air kerana enzim yang tersekat-gerak selalunya dalam keadaan kering. Ini sangat penting untuk lipase, kerana kebanyakan penggunaan lipase dalam industri adalah untuk sintesis ester dan pengubahsuaian lemak (transesterifikasi), yang hanya berlaku dalam kondisi air yang terbatas. Oleh itu, objektif penyelidikan ini adalah untuk menghasilkan satu kaedah yang mudah dan berkesan untuk menyekat-gerak lipase, dengan menggunakan sagu sebagai bahan sokongan dan menguji lipase yang tersekat-gerak tersebut untuk aktiviti esterifikasi dan transesterifikasi. Sagu adalah kanji yang diekstrak dari empulur pokok palma *Metroxylon sagu*. Ia dianggap sesuai sebagai bahan sokongan penyekat-gerakan kerana bersifat lengai, mempunyai struktur gel yang stabil and ketahanan yang sangat baik terhadap haba dan ricih.

Lipase termostabil yang dipencilkan dari *Geobacillus* sp. ARM (ARM lipase) digunakan dalam kajian penyekat-gerakan untuk menghasilkan kaedah yang mudah dan efisien untuk meningkatkan kestabilan enzim. Lipase ARM tulen digunakan untuk meminimumkan bahan asing yang mungkin mempengaruhi hasil eksperimen. Dua teknik penyekat-gerakan menggunakan bahan sokongan yang berbeza diuji, iaitu, penjerapan pada kitosan, amberlite, dan “layered double hydroxide” dan pemerangkapan di dalam sagu. Aktiviti lipase tertinggi

yang telah didapati adalah dengan pemerangkapan lipase dalam sagu yang digelatinkan yang kemudiannya dikering-sembur. Hasil tersebut disokong oleh analisis luas permukaan dan keliangan di mana luas permukaan, isipadu pori, dan radius pori masing-masing menurun sebanyak 67%, 50%, dan 10%, ketika lipase tersekat-gerak pada sagu, yang menunjukkan bahawa ruang tersebut telah diisi oleh enzim. Kaedah penyekat-gerakan ini telah meningkatkan kestabilan terma enzim dengan berkesan, di mana separuh hayat enzim pada suhu 80°C adalah 4 jam, berbanding dengan aktiviti enzim bebas, yang menurun dengan pantas dari jam pertama. Kaedah penyekat-gerakan ini juga berjaya mengekalkan enzim untuk jangka hayat yang lebih lama, iaitu 9 bulan pada suhu 10°C dan 2 minggu pada suhu bilik. Lipase ARM yang tersekat-gerak mengekalkan suhu optimum bagi aktiviti enzim pada 70°C dan pemilihan pH dari pH7 hingga pH9. Secara keseluruhan, hasil pencirian termal dan pH telah menunjukkan bahawa penyekat-gerakan lipase pada bahan sokongan semula jadi seperti sagu sangat berpotensi untuk kegunaan industri, terutamanya dalam aplikasi pembuatan makanan.

Dua aplikasi model dilaksanakan untuk menguji lipase yang tersekat-gerak menggunakan kaedah yang baru dihasilkan bagi aktiviti sintesis dalam persekitaran yang bebas air. Enzim yang tersekat-gerak digunakan untuk sintesis etil oleate dengan tindak balas esterifikasi dan sintesis alternatif mentega koko dengan tindak balas transesterifikasi (asidolisis dan interesterifikasi). Penggunaan lipase tersekat-gerak yang kering sangat relevan untuk aplikasi ini. Bagi eksperimen-eksperimen ini, enzim komersial, *Rhizopus oryzae* lipase (ROL), yang tersekat-gerak dengan menggunakan kaedah yang baru dihasilkan telah digunakan untuk perbandingan bersama lipase ARM. ROL yang tersekat-gerak menunjukkan hasil yang cemerlang bagi esterifikasi (81.3% pada 60°C) berbanding dengan ARM lipase (9.9% pada 60°C), oleh itu, ia telah digunakan untuk eksperimen yang selanjutnya. Penukaran etil oleat tertinggi yang diperoleh menggunakan ROL yang tersekat-gerak dari penyelidikan ini adalah 97% dengan pengeraman di dalam bekas air berkocak pada suhu 40°C dan 50°C, goncangan 200 rpm, selama 12 jam, dengan nisbah substrat etanol dan oleik asid 2:1. Bagi sintesis alternatif mentega koko menggunakan ROL yang tersekat-gerak dalam sagu, nisbah minyak sawit peringkat pertengahan, minyak zaitun, dan asid stearat yang berbeza digunakan sebagai substrat. Berdasarkan analisis komposisi kimia yang ditentukan oleh HPLC dan GCMS, produk yang menggunakan nisbah substrat 1:0:1 mempunyai bacaan yang terdekat dengan mentega koko berbanding dengan nisbah-nisbah lain, dengan 21.77% palmitoyl-oleoyl-palmitoyl glicerol, 27.71% palmitoyl-oleoyl-stearoyl glicerol, 13.39% stearoyl-oleoyl-stearoyl glicerol, jumlah asid lemak tepu 66.07%, dan asid lemak tak tepu sebanyak 33.93%. Bagi ciri-ciri terma pula, produk yang menggunakan nisbah substrat 1:1:1 mempunyai bacaan yang terdekat dengan mentega koko berbanding dengan nisbah-nisbah lain, dengan titik lebur gelincir 36.2°C; kandungan lemak pepejal sebanyak 7.73% dan 2.02% masing-masing pada suhu 30°C dan 40°C. Termogram DSC menunjukkan puncak lebur pada suhu yang lebih rendah daripada 37°C (11.56°C dan 33.41°C). Ringkasnya, kaedah penyekat-gerakan enzim yang baru dihasilkan menggunakan sagu sebagai bahan sokongan sesuai untuk lipase dari sumber bakteria dan kulat, untuk tindak balas kedepan dan balikan lipase.

ACKNOWLEDGEMENTS

Alhamdulillah, all praises and thanks to Allah, The Most Gracious and The Most Merciful. There is no power nor strength except by Allah.

The completion of this thesis was a very challenging journey for me, and I certainly could not have made it alone. I am forever grateful to my family for their endless love and support. Their patience and understanding deter me from giving up.

I would like to give special thanks to all my friends, especially from Enzyme Technology Lab, IBS, who have been together with me through thick and thin. They were a great company and always cheered me up, made a hellish life of a postgraduate bearable. To all who has helped me to the completion of this thesis, thank you very much.

Last but not least, I would also like to express my utmost appreciation to my supervisors Professor Dr Raja Noor Zaliha Raja Abdul Rahman, Professor Dr Mohd Basyaruddin Abdul Rahman, and Professor Dr Shuhaimi Mustafa, for their dedication, guidance and encouragement through the course of this research. A very special thanks to Dr Yanty Noorzianna and also the PIs in EMTech, I really appreciate their generous ideas and guidance. Last but not least, I would like to thank MyBrain 15 for sponsoring my PhD.

May Allah please, and may we always be in His blessings.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Raja Noor Zaliha Binti Raja Abd Rahman, PhD
Professor
Faculty Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd Basyaruddin Bin Abdul Rahman, PhD
Professor, ChM.
Faculty of Science
Universiti Putra Malaysia
(Member)

Shuhaimi Bin Mustafa, PhD
Professor
Faculty Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
Z**ALILAH BINTI MOHD SHARIFF, PhD**
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 10 June 2021

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Name and Matric No: Nur Syazwani Binti Mohtar

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Signature:	
Name of Chairman of Supervisory Committee:	Prof. Dr. Raja Noor Zaliha Raja Abd Rahman
Signature:	
Name of Member of Supervisory Committee:	Prof. Dr. Mohd Basyaruddin Abdul Rahman
Signature:	
Name of Member of Supervisory Committee:	Prof. Dr. Shuhaimi Mustafa

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× g	Gravitational force
A_{600}	Absorbance at 600 nm
bp	Base pair
CB	Cocoa butter
c/min	Centimeter per minute
cm/s	Centimeter per second
DNA	Deoxyribonucleic acid
EC	Enzyme classification
FAME	Fatty acid methyl ester
GC	Gas chromatography
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria-Bertani
LOL	Linoleoyl-oleoyl-linoleoyl glycerol
M	Molar
mg	Milligram
mL	Milliliter
mL/min	Milliliter per minute
mm	Millimeter
mM	Millimolar
N	Normal

NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
ng/mL	Nanogram per milliliter
OOO	Oleoyl-oleoyl-oleoyl glycerol
PDB	Protein Data Bank
PLO	Palmitoyl-linoleoyl-oleoyl glycerol
PLP	Palmitoyl-linoleoyl-palmitoyl glycerol
PMF	Palm mid-fraction
POO	Palmitic-oleoyl-oleoyl glycerol
POP	Palmitoyl-oleoyl-palmitoyl glycerol
POS	Palmitoyl-oleoyl-stearoyl glycerol
PPP	Palmitoyl-palmitoyl-palmitoyl glycerol
PPS	Palmitoyl-palmitoyl-stearoyl glycerol
PSS	Palmitic-stearoyl-stearoyl glycerol
ROL	*Rhizopus oryzae* lipase
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SLO	Stearoyl-linoleic-oleoyl glycerol
SLS	Stearoyl-linoleoyl-stearoyl glycerol
SOO	Stearoyl-oleoyl-oleoyl glycerol
SOS	Stearoyl-oleoyl-stearoyl glycerol
SSS	Saturated-saturated-saturated TAG

SUS	Saturated-unsaturated-saturated TAG
SUU	Saturated-unsaturated-unsaturated TAG
TAGs	Triacylglycerols
TLC	Thin layer chromatography
U	Unit
U/mg	Unit per milligram
U/mL	Unit per milliliter
UUU	Unsaturated-unsaturated-unsaturated TAG
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
µg/mL	Microgram per milliliter
µL	Microliter
µM	Micromolar
µmol	Micromole

CHAPTER 1

INTRODUCTION

Enzymes are proteins that act as catalysts for biochemical reactions and can be utilized to perform chemical reactions as well. Enzymes catalyze specific substrates and perform under milder conditions like temperature, pressure, and pH, in comparison to the conventional chemical catalysts (Filho et al., 2019). Therefore, enzymes usage for industrial processes is increasing. However, the main concern regarding the usage of enzymes that need to be addressed is its stability because enzymes are very fragile and at the risk of degradation with the slightest change in the environment like temperature, pH, and salt concentration. The studies to increase enzymes stability are expanding, from the modification of enzymes at the molecular level to the external protection on the enzyme, including the research on the apparatus used to perform the enzymatic reactions (Minteer, 2011). For industrial applications, simple methods to increase enzyme stability that are time and cost-efficient are more favorable, for example, using non-damaging instruments during the reactions, performing the reactions in mild conditions, adding soluble additives to the reactions, or chemically modify the enzymes using polymers like aldehydes, imidoesters, and anhydrides (Facin et al., 2019; Yabuki, 2017). However, those methods mainly address the concern of operational stability, but not the storage or shelf stability (Mohamad et al., 2015). A practical method that can tackle both operational and storage stability for the enzymes is to immobilize the enzymes on support materials.

Lipases are very versatile enzymes that are able to catalyze the hydrolysis of ester bonds as well as the synthesis of ester bonds. Therefore, lipases fit for applications in various industries, such as food processing, oleo-chemicals, detergent, dairy, pharmaceutical, cosmetic, biodiesel, and waste management (Guerrand, 2017). The market that employs microbial lipase was estimated at USD 425.0 million in 2018 and is projected to achieve USD 590.2 million by 2023 (Chandra et al., 2020). The potential of lipase can be increased by immobilization. Apart of the stabilizing effect, the immobilized lipase has many other advantages like easy to handle and store, easy to separate enzyme from products and reactants, fast termination of reactions, make the enzyme reusable and thus more economical. Also, the immobilization of lipase often leaves the enzyme in a dry form, enable the enzyme to perform the reverse reaction of lipase (esterification and transesterification) in an anhydrous condition.

In this research, two model reactions that involve the reverse reaction of lipase were focused on, that is, esterification for the synthesis of ethyl oleate and transesterification reaction for the synthesis of cocoa butter alternative. Ethyl oleate is mostly used as a solvent in pharmaceutical products that consists of lipophilic components, such as steroids. It can be used as food additives and

safe for consumptions (U.S. Food and Drug Administration, 2019). Ethyl oleate can be synthesized by esterification of ethanol and oleic acid. As for cocoa butter, it is a prominent fat in confectionery industries, especially chocolate. This is due to its organoleptic characteristics and physical properties that are important in various chocolate and confectionary productions. Other than in food industries, cocoa butter is also widely applied in cosmetics and pharmaceutical industries. The insecurity in supplies and the high price of cocoa butter have driven the search for an alternative cocoa butter-like fat. The global market value of cocoa butter alternatives is projected to reach USD 1.5 billion by 2024 with a strong compound annual growth rate of 5.1% forecasted within 2019 to 2024 (Market Research Future, 2020). Some of the attempts to produce cocoa butter-like fat are by enzymatic transesterification reaction using a cheaper fat or oil as substrate. The production of cocoa butter alternative could serve as a more economical option and a more stable supply.

For the purpose of this research, ARM lipase was used for the immobilization study. This enzyme was isolated from *Geobacillus* sp. strain ARM, a thermophilic lipolytic bacterium that has been sampled from soil contaminated with cooking oil in Taman Sri Serdang, Serdang, Selangor, Malaysia (Ebrahimpour et al., 2011). The ARM lipase is tolerant to organic solvent, optimum at pH 8.0 and 60°C, and prefers medium and long chain fatty acids as substrate (Ebrahimpour et al., 2011). This research is to develop a simple yet very effective method for enzyme immobilization. One of the candidates for immobilization supports in this study was sago. The main contributing factors for the selection of sago as the enzyme immobilization support were because it is cheap local produce that is inert and safe for consumption as it is derived from a natural source. Given that, the products using sago can be classified as biodegradable and eco-friendly. On top of that, sago also known to have a stable gel structure and have an excellent heat and shear resistance (Du et al., 2020). Lately, sago has been used in many experiments, such as prebiotics, composite film, nanoparticles, electrolyte, environmental cleaning agent, aquafeed, ceramic foam, emulsion formulation, pharmaceutical and medical applications, and lactic acid and pullulanase production (Zhu, 2019). Still, there are no studies published on sago as a support for enzyme immobilization to this date.

The significance of this study is to address the limitation of enzyme usage in harsh conditions, like high temperature and pH, and the presence of an organic solvent, that could threaten the stability of the enzyme. The immobilization of ARM lipase is hypothesized to increase its stability at high temperature for a longer duration and also for longer shelf-life compared to free lipase. Another hypothesis is that the immobilized lipase can perform the reverse reactions of lipase (esterification and transesterification) in a nonaqueous environment. In addition, a commercial lipase, that is, *Rhizopus oryzae* lipase (Lipase DF “Amano” 15, Amano enzyme) was also tested using the same immobilization method and used for esterification and transesterification reactions. Being isolated from a fungal source, *Rhizopus oryzae* lipase is classified in different lipase subfamily from ARM lipase. The most distinguishable feature between these two lipases is their structure, especially the lids where *Rhizopus oryzae*

lipase has one helical lid, and ARM lipase has two (Khan et al., 2017). The small lid of the fungal lipase did not fully cover its active site, making the enzyme easier to be activated, especially in an anhydrous reaction like esterification and transesterification where the oil-water interface is inadequate (Ortiz et al., 2019). Therefore, although both lipases share the same substrate preference, they might perform differently in different types of reactions (Borrelli & Trono, 2015). The use of lipases from various sources and different subfamilies was used as a proof of concept to test the efficiency of the newly developed immobilization method in performing lipase reactions in aqueous and nonaqueous conditions.

The following list summarized the problem statements that this research wants to address:

1. Enzymes are very sensitive and prone to degradation with the slightest change in the environment, like temperature, pH, and salt concentration.
2. Most of the supports used for immobilized enzymes in industries are synthetic rather than from natural and eco-friendly sources.
3. The majority use of lipase in the industry is for ester synthesis and fat modification (transesterification), which can only occur in a water-limiting condition. Free lipases that are usually produced in solution, either in culture media or in buffers, cannot perform the reverse reaction because water is present.
4. Although enzymes in the lipase family catalyze the same type of reaction, the properties of the enzymes like the molecular structure, substrate specificity, pH preference, thermostability, and reaction efficiency differ depending on their origins. Enzymes that have different structures might also react differently with the immobilization support or the immobilization technique. Therefore, the test on a single type of lipase cannot justify the effectiveness of the newly developed immobilization protocol, and so lipases from different subfamilies should be compared for confirmation.

The main objective of this research is to increase enzyme stability by a newly developed immobilization method and use it for esterification and transesterification reactions. The specific objectives are as follows:

1. To test several immobilization support materials (chitosan, amberlite, layered double hydroxide, and sago) by immobilizing purified ARM lipase using adsorption method and entrapment method.
2. To characterize the immobilized ARM lipase activity and stability.
3. To apply the newly developed immobilization method on a commercial lipase (*Rhizopus oryzae* lipase).
4. To compare the synthesis of ethyl oleate by esterification reaction of the immobilized ARM lipase and immobilized *Rhizopus oryzae* lipase.
5. To synthesis a cocoa butter alternative by transesterification using the immobilized *Rhizopus oryzae* lipase.

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