



A new isolate of thermophilic and organic solvent tolerant bacteria for lipase production using basal medium of palm kernel cake

Nurul Hidayah Mohd Zubairi, Md. Zahangir Alam*, Md Noor Salleh, Hamzah Mohd Salleh, Nurul Alia Fazil

Bioenvironmental Engineering Research Centre (BERC), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Kuala Lumpur, 53100, Malaysia.

Email: zahangir@iium.edu.my

ABSTRACT

Aims: This research focused on the selection of potential strains especially bacteria that can grow effectively in palm kernel cake (PKC) and produce high amount of thermostable and solvent tolerant (TS-OST) lipase. The work involved the exploration of renewable PKC as potential fermentation medium for discovery to novel TS-OST lipase that would have excellent tolerance and activity in presence of organic solvents with high temperatures for industrial applications.

Methodology and results: Using palm kernel cake (PKC) as source of thermophilic bacteria, 53 bacterial strains were found survived at temperature 65 °C. However, after subcultured several times, only 17 strains were found as pure thermophilic strains. Preliminary screening both qualitative and quantitative was performed to all 17 potential thermophilic bacterial strains and showed that only 11 purified thermophilic strains are lipase producer. Strain PKC-P1 produced highest enzyme activity (11.13 U/g), followed by PKC-P13 and PKC-C9. The lowest enzyme activity was lipase produced by PKC-C10 (0.76U/g). Strain PKC-P1 has been classified as Gram negative bacteria and identified as *Bacillus smithii* strain PKC_P1.

Conclusion, significance and impact of study: PKC as a by-product of oil palm industry consists of many nutrients that can give benefits towards industry and can be utilized in order to produce enzymes like lipases. From these results, it could be concluded that this lipase stable at temperature 65 °C and pH 7 and may be a potential candidate to be used in a variety of biotechnological applications. This finding revealed that a bacterial strain obtained from oil-rich environment which is PKC through isolation process has potential as a source of more economical enzyme to be applied in biotechnology industry.

Keywords: Palm kernel cake (PKC); lipase; Solid state fermentation (SSF); thermophilic bacteria; thermostable (TS); organic solvent tolerant (OST)

INTRODUCTION

Enzymes extracted from different sources: microbes, vegetables or animal organ have been used for ages in variety forms. A large number of enzymes are being produced and sold for various purposes and their production are getting more attention in life science industry sector. Nowadays, enzymes being used in multiple areas like food, feed, detergent, tanning, textiles, laundry, pharmaceuticals, cosmetics, and fine-chemicals industries and categorized based on specific applications. These industrial applications account for over 80% of the global market of enzymes (Whitehurst *et al.*, 2010). Over 500 industrial products are being made using enzymes (Johannes and Zhao, 2006; Kumar and Singh, 2013). The demand for industrial enzymes is on a continuous rise driven by a growing need for sustainable solutions. Microbes have served and continue to serve as one of the largest and useful sources of many enzymes (Demain and Adrio, 2008; Adrio and Demain, 2014). Lipase can be defined as carboxylesterases that catalyze hydrolysis and

synthesis of long chain acylglycerols each. They can be produced by various microorganisms like bacteria, fungi, archea and eucarya as well as plants and animals (Essamri *et al.*, 1998) and can be applied in various type of industry including pharmaceutical industries, food industries, cosmetics industries and others. In addition, lipases also have been used in biodegradation of plastics like polyhydroxyalkanoates (PHA) and polycaprolactone (PCL) (Imandi, 2010).

There are many reports on the production, purification and characterization of microbial lipases with single property of thermostable or organic solvent tolerant which limit the complete reactions in synthesis of biodiesel or biochemicals. Very few lipases have been reported having both characteristics such as thermostable and organic solvent-tolerant suitable in fermentation of solid wastes for industrial processes (Masomian *et al.*, 2013). Besides that, lipases can be used in the production of pharmaceuticals, cosmetics, leather, detergents, foods, perfumery, medical diagnostics, and other organic synthetic materials (Gandhi, 1997).

*Corresponding author

However, high cost of production of this enzyme remains the major limitation to large-scale industrial applications. In order to overcome this problem, the use of different microorganisms, supplements and substrates can contribute in getting the best combination to produce high value of lipases, using substrates and conditions that reduce the costs in industrial scale. Some reports on lipase production focused on solid substrates such as wheat bran, sugarcane bagasse, jatropha seed cake and water melon seeds (Treichel *et al.*, 2010). The use of cheap raw materials would diminish the operating costs of the process (Castilho *et al.*, 2000).

This research involved the exploration of renewable PKC as potential fermentation medium and establishing the necessary process conditions in a solid-state fermentation in the lab scale for discovery to novel TS-OST lipase that would have excellent tolerance and activity in presence of organic solvents with high temperatures for industrial applications. The work is conducted to evaluate the potential bacterial strains from palm kernel cake (PKC) with the properties of thermostable and organic solvent (TS-OST) lipase in PKC based medium in searching of enzymatic biodiesel production.

Organic solvent tolerant bacteria are group of microorganisms that can live in the presence of very high concentrations of organic solvents (Inoue and Horikoshi, 1989). Their potential in industrial and environmental biotechnology have been explored since the activity of the enzymes retain at a very high concentrations of organic solvents (Sardesai and Bhosle, 2004).

PKC is a solid waste obtained from palm kernel seeds after undergo the process of oil extraction (Chavalparit *et al.*, 2006), consists of many nutrients that can give benefits towards industry and can be utilized in order to produce enzymes like lipases. PKC was selected for microbial fermentation as it contains rich source of proteins as well as carbohydrates and (Imandi, 2010).

MATERIALS AND METHODS

Sample collection

Palm kernel cake (PKC) sample was collected from West Mill Sdn Bhd, Sime Darby Research Centre and compost sample was collected from International Islamic University Malaysia (IIUM).

Selection and purification of thermophilic strains

Approximately 0.1 g of the sample, PKC and compost were cultivated in 50 mL Luria-Bertani (LB) broth each, pH 7.0. Cultures were incubated at three different temperature; 55 °C, 65 °C and 70 °C 150 rpm for 48 h. In order to isolate bacteria with organic solvent properties, about 10% ethanol was added in the liquid media during cultivation. Sample of cultures were spread on the LB agar plate and incubated at each temperature (55 °C, 65 °C and 70 °C) for 48 h to identify thermophilic strains and

restreaked several times in order to obtain pure bacterial colonies.

Screening for lipase producing bacteria

Phenol red agar plate

Approximately 0.01% (w/v) of phenol red as well as lipidic substrates which are: 0.1% (w/v) CaCl₂, 1% (v/v) olive oil and 2% (w/v) agar were used in order to prepare phenol red agar. About 0.1 M NaOH was used to adjust pH. (pH7.3 - 7.4) (Singh *et al.*, 2006). Pure thermophilic strains got from above step will be streaked onto the prepared medium and incubated at room temperature for 48 h. Changing of agar colour was observed.

Tween-80 agar plate

Tween-80 considered as the most used substrates for the detection of lipase producing microorganisms in agar media (Emanuilova *et al.*, 1993). Tween-80 agar consists of some media which are: 0.5% (w/v) NaCl, 1% (w/v) peptone, 0.01% (w/v) CaCl₂·2H₂O, 2% (w/v) agar and autoclaved for 20 min. About 1% (v/v) Tween-80 was separately sterilized before pH of mixture was adjusted to pH 6.0 using 1 M NaOH. Overnight culture was spread on the agar and incubated at room temperature for 48 h. Observation towards the formation of visible precipitate will be used as an indication of lipolytic activity.

Inoculum preparation

Single colony for each lipase producing bacterial colony was transferred from LB agar plate into 20 mL sterile LB broth medium with pH 7 in 50 mL Erlenmeyer flask and incubated at 65 °C, 150 rpm for 18h. Inoculum was prepared with initial concentration of 10⁶.

Screening of potential bacterial strain for lipase producer

Fermentation process

Palm kernel cake was used as the basal medium. 6 g of PKC (moisture content of 70%) was added in the Erlenmeyer flask. Before proceeding to autoclave process, 1 M NaOH was used to adjust the initial pH to pH 6.0. About 2% (v/v) of inoculum was added in 150 mL Erlenmeyer flasks and was incubated for 48 h.

Enzyme extraction

The crude enzyme obtained from fermentation process was recovered by simple extraction method. Then, about 50 mL of distilled water was added into fermented substrate and the content was agitated for 2 h, room temperature at 150 rpm. The fermented media then was centrifuged at 8000 rpm, 4 °C for 20 min. Supernatants was collected and used as a lipase assays and biochemical characterization studies.

Lipase assay

A solution of 2.5 mmol of p-NPP was prepared in a Tris-HCl buffer. About 2.4 mL of this solution was added to 0.02 mL of crude lipase solution and was incubated at 65 °C for 15 min and cooled for 5 min. Then, absorbance of the solution was determined at 410 nm (Willerding *et al.*, 2012). This experiment was conducted in triplicate.

Bacterial strain identification

Gram staining method has been done on the selected strain (strain with highest lipase activity) by Burke method and confirmed by KOH test (Barron and Finegold, 1990). A few loopful of water was dropped on the slide and amount of colony was transferred aseptically. Culture was spread with inoculation loop and slide was moving gently on the flame to let it dry. Then, a drop of crystal violet was stained over the fixed culture and stand it for 10 to 60 seconds. Stain was gently rinsed with distilled water. Continued with addition a drop of iodine solution. After 10 to 60 seconds, slide was rinsed with distilled water and decolorized with 95% ethanol. Then a drop of safranin was added on the culture. After 45 seconds, slide was rinsed with distilled water. Cover slide was put on the culture once the slide dried and put under microscope to study the characteristics of the strain. Gel electrophoresis test and genotypic identification via determination the 16s rDNA sequence have been done. (Thompson *et al.*, 1994).

RESULTS AND DISCUSSION

Screening of thermophilic bacterial strain

After 48 h of incubation, colonies can be seen appeared on the plate incubated at 55 °C and 65 °C but not on the plate 70 °C. However, based on the observation more colonies appeared on the plate 65 °C compared to plate 55 °C. So, each colony on that plate was transferred on the fresh LB agar and incubated at 65 °C. After 24 h, only fifteen strains from compost source and thirty-eight strains from PKC source are appeared on the fresh LB agar plate. It showed that, they have characteristic of thermophilic strain as they can adapt at temperature 65°C as well as organic solvent tolerant as they can adapt at the present of 10% of organic solvent (ethanol). Thermophilic microorganisms are amongst the most studied extremophiles and their biocatalytic potential can be found in several studies (Vieille and Zeikus, 2001). Total of all 53 thermophilic strains then were restreak again on fresh LB agar plate and incubated at 65 °C and pure thermophilic strains were obtained. After 48 h, seventeen pure thermophilic strains from both sources were obtained as shown in Table 1. All these thermophilic

strains were grew optimally at 65 °C. No grow was observed above 70 °C. Attempts to isolate thermophilic bacteria from other sources like soil and water sample, which are capable of producing thermostable lipases have been started few decades ago (Nagaoka and Yamada 1973; Oi *et al.*, 1967; Watanabe *et al.*, 1977) found out only two strains selected as potent producers of alkaline lipase from total of 1606 isolated strains. Due to the fact that enzymes produced from thermophilic microorganism are well suited for harsh industrial process, they are gaining wide industrial and biotechnological interest. So, isolated thermophilic and organic solvent tolerant bacteria gained from this result having potential of producing thermostable and organic solvent tolerant lipase that can be applied in various industries.

Table 1: Purified thermophilic strains

Source	Strain code
Palm kernel cake	PKC-P13
	PKC-P12
	PKC-P10
	PKC-P52
	PKC-P53
Compost	PKC-P1
	PKC-C4
	PKC-C6
	PKC-C7
	PKC-C8
	PKC-C9
	PKC-C10
	PKC-C11
	PKC-C12
	PKC-C16
PKC-C14	
PKC-C15	

Qualitative test of lipase producing bacteria

Phenol red agar plate

This test was conducted to identify lipase positive strains on phenol red agar plate. Seventeen purified thermophilic strains were streak on the phenol red agar plate. As shown in Table 2, from all seventeen plates, it was observed that only eleven plates showed changing of color from pink to yellow (PKC-C4, PKC-C6, PKC-C7, PKC-C8, PKC-C9, PKC-C10, PKC-C11, PKC-C12, PKC-C16, PKC-P1 and PKC-P13). A color change determined the existence of lipase enzyme. Figure 1 shows best three strains that completely change phenol red agar plate from pink to yellow. Strains PKC-P12, PKC-P10, PKC-P52, PKC-P53, PKC-C14 and PKC-C15 did not produced lipase. They are not lipase producer. It proved that only these eleven thermophilic strains are lipase producer. The changing of color for phenol red agar plate from pink to yellow indicates the decrease in pH due to release of fatty acids on lipopolysis.

Table 2: Result of preliminary qualitatively screening for lipase producing bacteria.

Strain code	Remarks
PKC-P1	Phenol red agar completely change from pink to yellow indicates that strain PKC-P1 produced large amount of lipase. However, no changing occurred for Tween-80 agar plate. This strain gives no reaction on this plate.
PKC-C4	Phenol red agar slightly change from pink to orange as well as formation of white precipitate for Tween-80 plate indicates that strain PKC-C4 produced small amount of lipase.
PKC-C6	Phenol red agar completely change from pink to yellow indicates that strain PKC-C6 produced large amount of lipase. However, no changing has been detected for Tween-80 agar plate. This strain gives no reaction on this plate.
PKC-C7	Phenol red agar completely change from pink to yellow indicates that strain PKC-C7 produced large amount of lipase as well as formation of white precipitate for Tween-80 agar plate proved the existence of lipase.
PKC-C8	Phenol red agar slightly change from pink to orange indicates that strain PKC-C8 produced small amount of lipase and white precipitate formed on the Tween-80 agar plate indicates the presence of lipase
PKC-C9	Phenol red agar slightly change from pink to orange indicates that strain PKC-C8 produced small amount of lipase and there is no lipase have been detected on the Tween-80 agar plate.
PKC-C10	Slightly change from pink to orange color occurred on the phenol red agar plate indicates that only small amount of lipase were produced and no detection for Tween-80 agar plate.
PKC-C11	Phenol red agar change to yellowish-orange indicates that the lipase produced are not as much as lipase produced by strain PKC-C1 but more than lipase produced by strain PKC-C4. However n detection for tween-80 agar plate.
PKC-C12	Strain PKC-C12 produced high amount of lipase since the color of phenol red agar completely change from pink to yellow as well as formation of white precipitate on Tween-80 agar plate.
PKC-C16	Strain PKC-C16 produced high amount of lipase since the color of phenol red agar completely change from pink to yellow as well as formation of white precipitate on Tween-80 agar plate.
PKC-P13	Strain PKC-P13 produced lower amount of lipase compared to lipase produced by strain PKC-C16 since the color of phenol red agar not completely change from pink to yellow. However, lipase produced were detected by Tween-80 agar.
PKC-P12	No lipase have been produced
PKC-P10	No lipase have been produced
PKC-P52	No lipase have been produced
PKC-P53	No lipase have been produced
PKC-C14	No lipase have been produced
PKC-C15	No lipase have been produced



Figure 1: Phenol red agar tests: a) Strain PKC-P1, b) Strain PKC-C12, c) Strain PKC-C6.

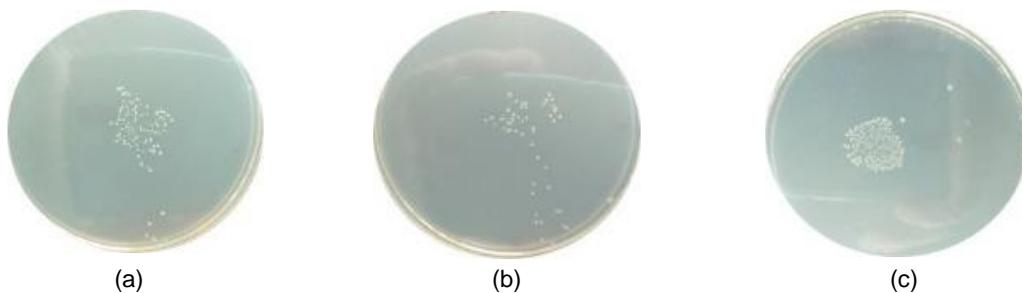


Figure 2: Tween-80 agar tests: a) Strain PKC-P1, b) Strain PKC-C7, c) Strain PKC-

Tween-80 agar plate

White precipitate has been observed only on five plates (PKC-C7, PKC-C8, PKC-C12, PKC-C16, and PKC-P13) as stated in Table 2. Figure 2 shows best three Tween-80 agar plates with formation of white precipitate. This formation occurred by dismissal of insoluble calcium crystal salt by the relief of the fatty acid as the bacteria grow on the Tween-80 agar plates. The others strain might be not a lipase producer or lipase produced by these strains were not detected by Tween-80 agar plate.

Quantitative test of lipase producing bacteria

Table 3: Enzyme activity (Unit/g) of lipase produced.

Strain Code	Enzyme activity (Unit/g)
PKC-P13	10.78
PKC-C16	4.45
PKC-C4	4.83
PKC-P1	11.13
PKC-C11	4.14
PKC-C6	7.20
PKC-C10	0.76
PKC-C9	10.20
PKC-C12	9.40
PKC-C7	8.04
PKC-C8	2.67

Potential lipase producer bacteria screened from step before were further screened to confirm amount of lipase activity for each strain. Palm kernel cake was used as medium since agricultural waste like palm kernel cake contained substantial amount of fatty acid and nutrients. This method considered as quantitative screening of lipase producing bacteria. Enzyme activities of lipase produced by all eleven strains were observed through p-nitrophenylpalmitate (pNPP) assay. From Table 3, it showed that strain PKC-P1 produced highest enzyme activity (11.13 U/g), followed by PKC-P13 and PKC-C9. The lowest enzyme activity was lipase produced by PKC-C10 (0.76 Unit/g). Lipases produced having characteristics of thermostable and organic solvent tolerant as they can withstand temperature 65 °C with the present of organic solvent. Nowadays, organic solvent tolerant lipase gets more attention as they have ability to markedly improve the production of biodiesel. Some studies found that some lipases especially those came from genera *Pseudomonas* and *Bacillus*, have ability to tolerate ethanol and methanol and hence could be applied for biodiesel production, from low cost precursors and waste lipids (Fang *et al.*, 2006). The successful isolation of eleven thermal and organic solvent tolerant bacterial strains with capability of producing lipase enzymes proved

that PKC has potential to act as an oil and lipid rich environment for bacterial growth. Strain PKC-P1 shows highest lipase activities, both qualitatively and quantitatively which therefore selected as the potential strain for the subsequent lipase production. Characteristics of this strain and its genotypic identification have been studied.

Identification of thermophilic strain

Gram staining method has been done on selected strain, PKC-P1 due to its high lipase activity. The characteristic of the strain has been identified as states in Table 4. Strain PKC-P1 has been classified as gram negative bacteria with rod shape. Identification of thermophilic bacteria was further confirmed by NCBI 16S ribosomal RNA sequences method.

Bacterial identification by NCBI 16S ribosomal RNA sequences

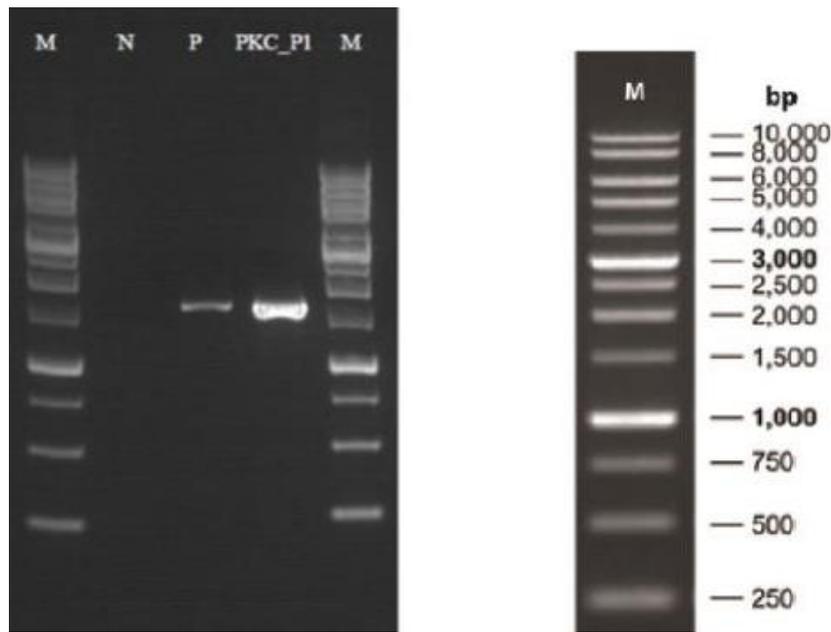
According to gel electrophoresis test, strain PKC_P1 has base pair of 1.5 kb full length as shown in Figure 3. 16 rRNA gene sequence of the strain PKC_P1 was analyzed. The BLAST algorithm was used to retrieve for homologous sequences in GenBank to the obtained 16S rRNA sequence. The bacterial isolated revealed 99% identify to full genome of *Bacillus smithii* strain DSM 4216. A phylogenetic tree of homologous family of *Bacillus smithii* strain PKC_P1 presented in Figure 4. Study on thermophilic *Bacillus* strains have been done by (Ronimus *et al.*, 1997). This study proved that *Bacillus* strains are mesophilic and thermophilic as they are able to grow at temperature 37 °C to 75 °C. *Bacillus smithii* known as facultative anaerobe and facultatively thermophilic bacterium (Bosma *et al.*, 2016). It has biotechnological potential, as it is able to ferment a range of carbon sources (Nakamura *et al.*, 1988) into lactate and other green building block chemicals (Bosma *et al.*, 2015).

CONCLUSIONS

This study was conducted to explore the capability of abundant waste product which is PKC as a source of thermophilic and organic solvent tolerant bacteria as well as a basal medium for bacterial growth through solid state fermentation (SSF) process. The product obtained, lipase with characteristics: thermostable and organic solvent tolerant was the target of this study due to its applicability to be applied in biotechnology industries. PKC was selected to be used in this study since it contained a lot of nutrients that may contribute benefit to the industry.

Table 4: Gram staining of strain PKC-P1.

Strain code	Color	Shape	Type of Gram	Microscopy analysis (40x)
PKC-P1	Pink	Rod	Gram (-)	



N: PCR no-template control (water to replace DNA template)
 P: Positive control (DNA extracted from *E. coli* is used as template)

Figure 3: Gel electrophoresis for thermophilic and organic solvent tolerant bacteria strain PKC_P1.

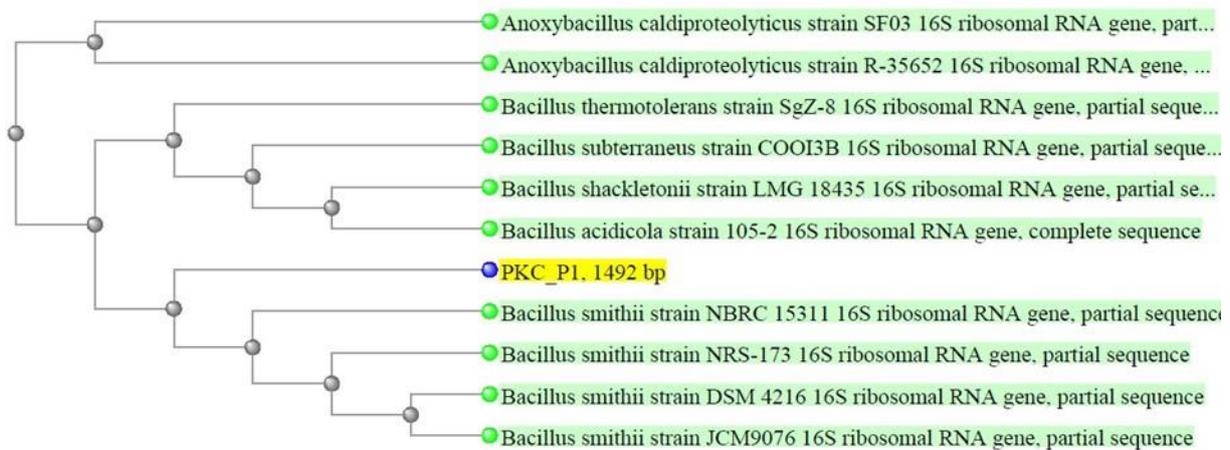


Figure 4: Phylogenetic tree-neighbor joining of the homologous family of *Bacillus smithii* sp. PKC_P1.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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