การคัดเลือกยีสต์จากทะเลและป่าชายเลนที่มีประสิทธิภาพในการผลิตเอนไซม์ไลเปส

Selection of lipases-producing yeasts from mangrove and marine environments

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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อแยกยีสต์ที่มีประสิทธิภาพสูงในการผลิตเอนไซม์ไลเปสโดยใช้แหล่งคาร์บอนชนิดต่าง ๆ เป็นซับสเตรท นำยีสต์ 73 สายพันธุ์ที่แยกได้จากป่าชายเลน น้ำทะเล และตะกอนทะเลด้วยวิธี enrichment มาคัดเลือกการผลิตเอนไซม์ไลเปสบนอาหารแข็งที่มีไตรบูไทรินและทวีน 80 เป็นองค์ประกอบด้วยวิธี plate assay ผลการทดลองพบว่ามียีสต์ 60 สายพันธุ์ที่แสดงบริเวณใสบนอาหารแข็งไตรบูไทริน และ 10 สายพันธุ์ที่แสดงบริเวณขุ่นบนอาหารแข็งทวีน 80 โดยสายพันธุ์ SPA4-2 มีค่าประสิทธิภาพของเอนไซม์ (enzyme activity index, EAI) สูงสุด เท่ากับ 3.38 บนอาหารแข็งไตรบูไทริน ขณะที่สายพันธุ์ BP12 มีค่า EAI สูงสุดเท่ากับ 2.16 บนอาหารแข็งทวีน 80 จากการศึกษากิจกรรมเอนไซม์ไลเปสของยีสต์สายพันธุ์ SPA4-2 และ BP12 ในอาหารเหลวที่มีแหล่งคาร์บอนชนิดต่าง ๆ ได้แก่ ไตรบูไทริน ทวีน 80 น้ำมันปาล์ม และน้ำมันใช้แล้วเป็นแหล่งคาร์บอนที่มีความเข้มข้นเริ่มต้นเท่ากับ 20 กรัมต่อลิตร ผลการทดลองพบว่าสายพันธุ์ SPA4-2 และ BP12 มีกิจกรรมของเอนไซม์ไลเปสสูงสุดในอาหารที่มีน้ำมันปาล์มเป็นแหล่งคาร์บอน โดยมีกิจกรรมของเอนไซม์ไลเปสที่เวลาเพาะเลี้ยง 120 ชั่วโมง เท่ากับ 1.53±0.11 และ 0.75±0.06 units/mL ตามลำดับ ยีสต์สายพันธุ์ SPA4-2 ที่ผลิตเอนไซม์ไลเปสได้สูงสุดถูกจัดจำแนกเป็น *Cryptococcus liquefaciens* ซึ่งมีความเหมือนกับ *C. liquefaciens* CBS 968^{T} (AF181515) 99.83 เปอร์เซ็นต์ จากผลการทดลองแสดงให้เห็นว่าสายพันธุ์ SPA4-2 มีความเหมาะสมต่อการผลิตเอนไซม์ไลเปสและจะเป็นประโยชน์ต่อการนำไปใช้ในระดับอุตสาหกรรมต่อไปในอนาคต

ABSTRACT

In order to isolate highly efficient lipase producing yeast, the present study aimed to investigate lipase production by using various carbon source as the substrates. Seventy-three yeast strains were isolated from mangrove forests, sea water and sea sediments by enrichment technique. These strains were screened for lipase production on agar medium containing tributyrin and Tween 80 by plate assay method. A clear zone on tributyrin solid medium was observed from 60 yeast strains, and 10 strains showed opaque zone on Tween 80 solid medium. The enzyme activity index (EAI) of strain SPA4-2 was sharply high value on tributyrin medium (EAI=3.38) whereas BP12 strain was effective highly value on Tween 80 medium (EAI=2.16). The lipase activity of the yeast strains SPA4-

2 and BP12 were also estimated in media containing various carbon sources such as 20 g/L each of tributyrin, Tween 80, commercial palm oil and waste cooking oil. The results implied that extracellular lipase activity of SPA4-2 and BP12 after incubation at 120 h in medium supplemented with commercial palm oil were 1.53±0.11 and 0.75±0.06 units/ mL, respectively. The yeast strain SPA4-2 that produced high lipase activity was identified as *Cryptococcus liquefaciens* with 99.83% sequence similarity to *C. liquefaciens* CBS 968^{T} (AF181515). These results indicated the strain SPA4-2 was suitable for lipase production and will be utilized in the industries in the future.

คำสำคัญ: การแยก บริเวณ D1/D2 ของ LSU rRNA gene ยีสต์จากป่าชายเลน ยีสต์ทะเล เอนไซม์ไลเปส
Keywords: Isolation, D1/D2 region of LSU rRNA gene, Mangrove forest yeasts, Marine yeasts, Lipases

INTRODUCTION

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are serine hydrolases which hydrolyze triglycerides to free fatty acids and glycerol by cleaving of the ester bond. Generally, these enzymes are widely used in the industries especially in a process of esterification or transesterification (Yucel et al., 2012; Aarthy et al., 2014). They are frequently used in various industrial sectors *viz.* detergent formulations, oleochemical industry, biofuel, dairy food industry, agro-chemical, paper manufacturing, nutrition, cosmetics industry and pharmaceuticals industry. Microbial lipases are usually obtained from bacteria, mold and yeast. Particularly, yeast and mold, which are important source of lipases for industrial application (Sharma and Kanwar, 2014). Most commercially important lipase-producing yeasts belong to the class of ascomycetous yeast such as *Candida* and the enzyme are extracellular (Anna et al., 2007). Many lipase-producing yeasts are previously reported, however only a few have been commercially exploited in production such as *C. antarctica*, *C. rugosa*, *C. tropicalis*, *C. cuvata*, *C. cylindraceae*, *C. deformans*, *C. parapsilosis*, *C. utilis*, *C. valida*, *C. viswanathii*, *Galactomyces geotricum*, *Arxula adeninivorans*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Trichosporon fermantans*, *Trichosporon asahii*, *Rhodotorula mucilaginosa* and *Aureobasidium pullulans* (Ciafardini et al., 2006; Potumarthi et al., 2008; Thakur, 2014). Fabiszewska et al. (2015) had reported extracellular lipase activity and lipase production by *Y. lipolytica* on glycerol medium were 0.1 unit/mL and 37.5 g/L, respectively. Moreover, *C. guilliermondii* presented high enzymatic activity (26.8±1.5 unit/mL) through submerged fermentation using agroindustrial residues as culture medium (Oliveira et al., 2014). Therefore, lipase-producing yeast can potentially and commercially be a good source of enzyme for industrial use.

Thailand is a tropical country rich in microbial diversity especially yeasts. Many newly identified yeast species have been proposed such as *Candida konsanensis* sp. nov. and *Occultifur tropicalis* f.a., sp. nov. (Sarawan et al., 2013; Khunnamwong et al., 2015). Some strains could produce primary and secondary metabolites *viz.* ethanol (from *Pichia kudriavzevii* PBB511-1), lipid (from *Trichosporon asahii* GSY10), and idole-3-acetic acid (from *Rhodosporidium paludigenum* DMKU-RP301) (Kaewkrajay et al., 2014; Nutaratat et al., 2015; Paserakung et al., 2015). However, these strains were isolated from terrestrial habitat. Although mangrove and marine habitats are rich in microorganisms, these niche are still less studied. Even though the superior metabolites producing could be found from them. They are potential sources of lipids, including omega-3-fatty acid, many types of enzyme

and also high ability to enhance prevention on postharvest decay of fruits (Wang et al., 2011; Gupta et al., 2012; Duarte et al., 2013). Thus these areas are more interesting to study.

Many carbon sources are considered as a feedstock to lipase production such as tributyrin, Tween 80, olive oil, glucose, glycerol, oil crops, animal fat, waste cooking oil and soybean oil etc. (Liu et al., 2014; Oliveria et al., 2014; Yalcin et al., 2014). Glycerol, oil crops and waste cooking oil were especially considered as the alternative feedstock to produce lipases for biodiesel production. Therefore, tributyrin, Tween 80, commercial palm oil and waste cooking oil were candidate for lipase production in this research which could be extended for biodiesel production in the future.

The aim of this research is to isolate the yeast strains from mangrove forests and marine habitats by screening for lipase production prior to identification by morphological and molecular characteristics. Selected yeast strains were grown in the basal medium which contained various carbon sources for analysis of enzyme activity.

MATERIALS AND METHODS

Sampling and isolation

Sampling: Soil, water, decaying wood, leaf and fruit were sampled from mangrove forests at Bangpoo district, Samutprakarn province and the nature education center for mangrove conservation and ecotourism in Chonburi. Seawater and marine sediments were collected from Sattahip Sea, Chonburi province. All samples were kept in plastic bag and/or sterile glass bottles and were then immediately transported to the laboratory for isolation.

Isolation: Two grams of soil samples and 5 mL of water samples were each suspended in a 250 mL Erlenmeyer flask containing 50 mL yeast extract-malt extract (YM) which was supplemented with 300 mg/L of penicillin, and 300 mg/L of streptomycin to inhibit bacterial growth and 0.025% propionate to suppress filamentous fungi. The suspension were then incubated in an incubator shaker with shaking speed of 150 rpm at 30 °C for 48 h. One loop of the cells was cross-streaked on YM agar plates and incubated at 30 °C for 48 h. Pure cultures were obtained from subsequent sub-culturing. Each pure cultures plates were transferred onto YM agar slants and kept at 4 °C.

Plant samples (decaying wood, leaf and fruit) were cut into smaller size of 0.5 x 0.5 cm with weight 2 g. These small fragments were rinsed twice with sterile normal saline and then dried on sterile filter paper before transferring into the medium. The isolation was done as previously mentioned. Isolation from seawater and marine sediments from Sattahip Sea were conducted similarly to the mangrove samples with the exception that the medium used was supplemented with 3.8 g/L of sea salt.

Screening for lipase-producing yeasts

Preliminary screening for lipase-producing yeasts was performed using plate assay with modified methods from Yalcin et al. (2014) and Oliveria et al. (2014). Briefly, the pure cultures were cultivated on YM agar plates and incubated at 30 °C for 24 h prior to inoculation on solid medium containing 10 g/L tributyrin, 23 g/L tributyrin agar with adjusted initial pH of 7.0 and 10 g/L Tween 80 in YM medium with adjusted initial pH of 7.0 by sterile toothpick. The plates were incubated at 30 °C for 120 h. The diameter of the reaction zone and colony were measured by Vernier caliper. The enzyme activity index (EAI) was calculated based on the ratio of the diameter of reaction zone to diameter of the colony.

Lipase production

One loop full of selected yeast was transferred to 50 mL of YM medium in 250 mL Erlenmeyer flask and cultivated at shaking speed of 160 rpm at 25 °C for 24 h. The culture was harvested and the optical density was measured with spectrophotometer at the wavelength 600 nm. Optical density was adjusted to 0.5 and transferred to 50 mL of the production medium which contained 20 g/L or either tributyrin, Tween 80, commercial palm oil or waste cooking oil, 3 g/L of yeast extract, 5 g/L of peptone, 1 g/L of $MgSO_4 \cdot 7H_2O$, 1 g/L of KH_2PO_4 and 1 g/L of $NaNO_3$. The medium was adjusted to the initial pH of 7.0. Cells were grown by shaking speed of 160 rpm at 25 °C for 120 h (Duarte et al., 2013; Treichel et al., 2010). The experiments were performed in triplicate and each aliquot was sampled at 48 and 120 h for analysis of enzyme activity and growth.

The samples were centrifuged at speed of 15000 rpm at 4 °C for 10 min. The supernatant which contained crude enzyme was preserved at -20 °C until enzyme activity was analyzed and the yeast cells were measured by spectrophotometer (OD600nm).

Identification of yeast

The morphology and molecular taxonomy were studied in order to identify lipase-producing yeasts. The nucleotide sequences of D1/D2 region of large subunit (LSU) rRNA gene were obtained with primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG- 3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G- 3'). Firstly, the DNA (deoxyribonucleic acid) from yeast cells was extracted by boiling in hot water and the target gene amplified by PCR. Secondly, the PCR product was checked by agarose gel electrophoresis and purified using kit, according to the manufacturer's protocol. Then, the purified products were submitted to Macrogen (Korea) for sequencing with the primer NL1 and NL4 for the D1/D2 region of LSU rRNA gene. Finally, the sequences were compared pairwise using the BLAST search program and were aligned with the sequences of related species which were retrieved from GenBank using CLUSTAL_X version 1.81 program (Tamura et al., 2011).

Analytical methods

The samples were centrifuged at 15000 rpm at 4 °C for 10 min. The supernatant was analyzed for enzyme activity and the yeast cells were measured by the methods as follow.

Growth: the yeast cells were washed twice with distilled water and then suspended with distilled water before reading the optical density by using spectrophotometer (PerkinElmer UV/Vis Lambda 25) at the wavelength 600 nm.

Enzyme activity: p-nitrophenyl palmitate (pNPP) was used as the substrate. pNPP (30 mg) was dissolved in 10 mL propanol which were emulsified in 90 mL of 50 mM Sodium acetate, pH 5.6, containing 200 mg Triton X-100. 100 µL of crude enzyme was mixed with 2 mL of pNPP-containing emulsion and then incubated at 37 °C for 15 min. The reaction was stopped by adding 150 µL of 1M Sodium carbonate solution and the absorbance was measured by spectrophotometrically at 410 nm against an enzyme-free control. One lipase unit (U) was defined as the release of 1 µmol of pNP per minute (Yalcin et al., 2014).

Statistical analysis: Data were analyzed through one-way ANOVA and post-hoc test ($p<0.05$) to consider as statistically significant.

RESULTS AND DISCUSSION

Yeast isolations

Yeast strains were isolated from 44 samples collected from mangrove forests and 26 marine samples (sea water and sea sediments). A total of 73 yeast strains (50 strains from mangrove forests and 23 strains from the marine samples), were obtained (Table 1).

Table 1 Yeast isolation from mangrove forests and marine habitats

Habitats	Yeast strains
Mangrove forest	BPS1, BPW1, BPF1, BPS2, BPL2, BPS3, BPW3, BPL3, BPS5, BPW5, BPS6, BPW6, BPS7, BPS8, BPS9, BPS10, BP12, BP13, BP16, BP17, BP18, BP19, BP20, BP21, BPS22 CH1, CHW1, CHC1-1, CHC1-2, CH2, CHW2, CH3, CH4, CHW4, CH5, CH6, CH8, CH9, CH10, CH11, CH12, CH13, CH14, CH15, CH18, CH20, CH21, CH22, CH23, CH24
Marine	SO6, SO7, SO14-1, SO19-1, SO19-2, SO22-1, SO25, SPA2-1, SPA3-1, SPA4-1, SPA4-2, SPA4-3, SPA4-4, SPA4-5, SPA7-1, SPA8-1, SPA11-1, SPA11-2, SPA11-3, SPA11-4, SPA11-5, SPA12-1, SPA13-1

The different colonial characteristic on YM agar plates were observed 0 to 3 isolates per one sample. Tantirungkij et al. (2012) reported that the total amounts of yeasts 365 isolations were investigated from 150 samples of sediments of salterns and mangrove as well as guts of marine animals by using enrichment culture technique. The different yeast colonial characteristic was found 1 to 4 types and all most of 90% was found 1 to 2 types per sample.

These yeast strains were preserved at -80°C by adding 10% glycerol and also kept at 4 °C on YM agar slant for further study.

Screening for lipase-producing yeasts

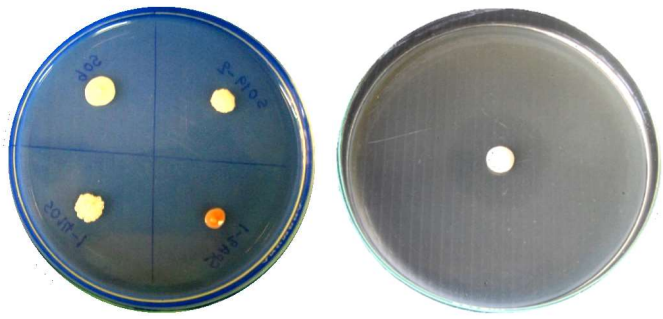
Preliminary screening lipase-producing yeasts were observed on agar plates which contained tributyrin and Tween 80. The clear zone or opaque zone was shown as Figure 1. The results showed 60 and 10 yeast strains could hydrolyze tributyrin and Tween 80, respectively. The results also showed 11 strains were reactive on both medium (Table 2). In addition, the strain SPA4-2 showed the highest EAI value on tributyrin agar (EAI = 3.38) and the strain BP12 showed the highest EAI value on Tween 80 agar (EAI = 2.16).

Almost all yeast strains could hydrolyze tributyrin but only a few strains could hydrolyze Tween 80. The chemical structure of these materials showed a different carbon number (tributyrin is C_4 while Tween 80 is C_{18}). Moreover, Tween 80 is emulsified properties which contained complex compounds, so it might be showed unclear opaque zone. Thus the enzyme activity of yeast strains SPA4-2 and BP12 were determined in broth medium.

In 2012, Tantirungkij et al. observed clear zone and opaque zone on a modified marine agar (MA) medium which contained tributyrin and Tween 80. The clear zone, opaque zone and colony diameter were measured after incubated at 30 °C for 48 h and then the lipase activity index was calculated. The results showed 261 isolates hydrolyzed Tween 80 and 116 isolates hydrolyzed tributyrin. The yeast strains could produce lipases and recorded a lipase activity index of ≥ 2 on both media. The highest lipase activity index on MA-Tween 80 was observed from strain T22 (LAI = 3.8) whereas strain T58 has the highest lipase activity index on MA-tributyrin (LAI = 2.5).

Moreover, many research were reported to screen lipases-producing yeasts which isolated from different sources such as active sludge, soil and wastewater samples from petroleum refinery, soil contaminated by petroleum, marine environments and castor leaves (Wang et al., 2007; Oliveria et al., 2014). In 2014, Yalcin et al. reported that 120 yeast isolates were obtained from environments contaminated with petroleum. Among these isolates, 12 were lipase-producing yeasts named as D3, D17, D24, D27, D30, D38, D40, D42, D44, D46, D56 and D57 and clear zone diameter was 3, 4, 4, 3, 4, 5, 5, 4, 7, 8, 3, 5, 5 and 6 mm., respectively. In addition, total 427 yeast strains were isolated from seawater, sediments, mud of salterns, guts of the marine fish and marine algae. The lipase activity of these yeasts was estimated by cultivation in the medium which contained olive oil. The results found that nine yeast stains could produce lipase and identified as *Candida intermedia* YA01a, *Pichia guilliermondii* N12c, *Candida parapsilosis* 3eA2, *Lodderomyces elongisporus* YF12c, *Candida quercitrusa* JHSb, *Candida rugosa* wl8, *Yarrowia lipolytica* N9a, *Rhodotorula mucilaginosa* L10-2 and *Aureobasidium pullulans* HN2.3. The optimal pH and temperature of lipase production were between 6.0 and 8.5 and between 35 °C and 40 °C (Wang et al., 2007).

For preliminary screening by plate assay, the results showed that the yeast strain SPA4-2 and BP12 could produce lipases and displayed highest EAI value on solid medium containing tributyrin and Tween 80, respectively. Thus both of yeast strains were selected for further study in broth medium for quantitative analysis of enzyme activity.



(A) (B)

Figure 1 Lipases producing test by plate assay on solid media containing 10 g/L tributyrin and Tween 80 (A) and clear zone diameter on tributyrin after incubation at 30 °C for 120 h (B).

Lipase activity of selected yeast strains

The yeast strains SPA4-2 and BP12 were selected for examination of enzyme activity by cultivation in broth medium contained various carbon sources such as tributyrin, Tween 80, commercial palm oil and waste cooking oil and cultivated in incubator with shaking speed of 160 rpm at 25 °C for 120 h. Both strains showed increased amount enzyme activity in commercial palm oil as the carbon source. After 48 h of the incubation period, the strain SPA4-2 and BP12 presented 1.09±0.27 and 0.03±0.04 units/mL and after 120 h, the enzyme activity showed 1.53±0.11 and 0.75±0.06 units/mL, respectively (Figure 2A, B). In term of growth rate, the optical density (600nm) of SPA4-2 was 13.75±4.20 and 20.83±6.93 while BP12 was 22.81±2.60 and 65.25±8.52 after incubation for 48 and 120 h, respectively (Figure 3A, B). The main fatty acid content of palm oil are palmitic acid (43.5%) and oleic acid (36.6%). In 2011, Srimhan et al. proposed that lipase- producing yeasts exhibited the ability to

catalyzed esterification reaction of oleic acid. *R. mucilaginosa* P11I89 showed the strongest hydrolytic lipase activity of 1. 2 units/ mL against palm oil. Therefore, palm oil can be a suitable feedstock for lipase production.

Table 2 Enzyme activity index (EAI) on solid media containing 10 g/L tributyrin and Tween 80.

strain	Enzyme activity index (EAI)	
	Tributyrin	Tween 80
BPS1	1.32±0.07	1.37±0.05
BPW1	1.37±0.10	1.28±0.01
BPS2	1.28±0.12	1.23±0.00
BPS7	1.48±0.03	1.18±0.04
BP12	1.38±0.06	2.16±0.10
BP17	1.47±0.04	1.27±0.08
BP19	1.48±0.17	1.12±0.06
SO25	1.27±0.08	1.21±0.00
SPA2-1	3.03±0.05	1.45±0.00
SPA4-2	3.38±0.00	1.20±0.01
SPA12-1	1.43±0.02	1.20±0.00

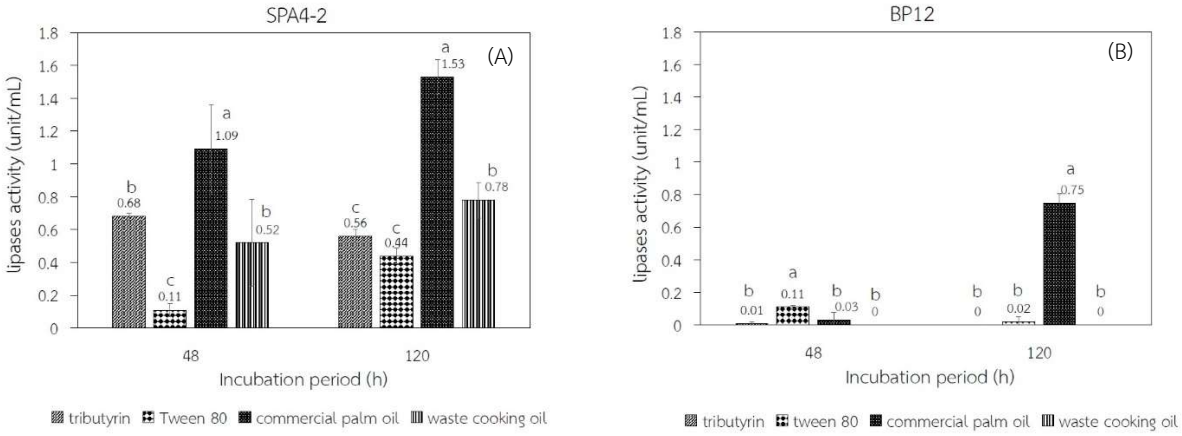


Figure 2 Lipase production of SPA4-2 strain (A) and BP12 strain (B) in broth medium contained 20 g/L of tributyrin, Tween 80, commercial palm oil and waste cooking oil, incubated at 25 °C with shaking speed of 160 rpm for 120 h.

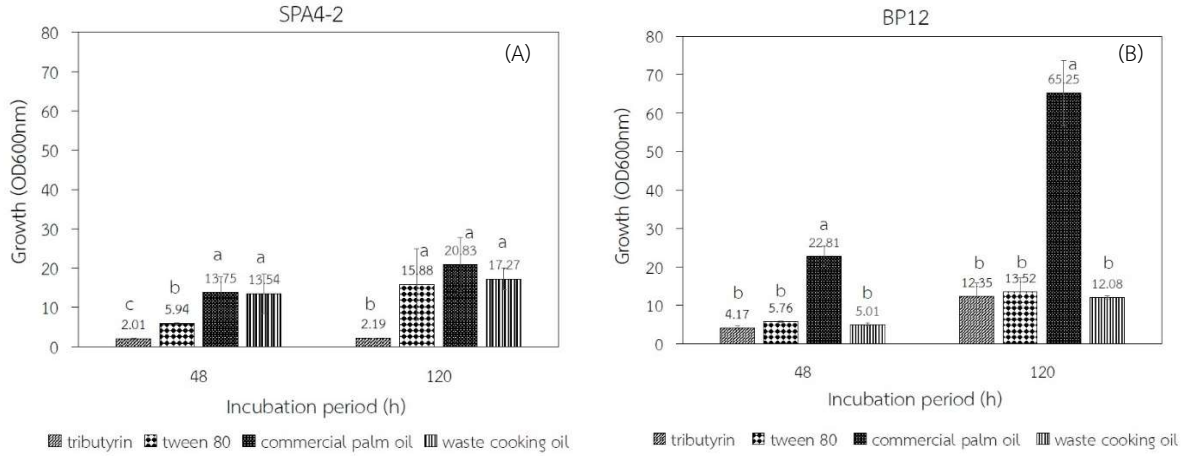


Figure 3 Growth of SPA4-2 strain (A) and BP12 strain (B) in broth medium contained 20 g/L of tributyrin, Tween 80, commercial palm oil and waste cooking oil, incubated at 25 °C with shaking speed of 160 rpm for 120 h.

Other carbon sources (tributyrin, Tween 80 and waste cooking oil) were hydrolyzed by yeast strain SPA4-2. The results showed enzyme activity were 0.56±0.04, 0.44±0.05 and 0.78±0.11 units/mL after 120 h whereas the enzyme activity at 48 h was 0.68±0.02, 0.11±0.04 and 0.52±0.26 units/mL, respectively. In case of growth, the results showed high optical density (OD600nm) were 2.19±0.05, 15.88±9.06 and 17.27±2.78 at 120 h and 2.01±0.09, 5.94±0.19 and 13.54±5.05 at 48 h. These results revealed that only tributyrin which supplemented in the broth medium did not present high amount of enzyme activity, although increasing of incubation time because of the low concentrated of yeast cells. Thus the optimization of utilization of each carbon sources are necessary for further study. Particularly, strain BP12 showed minute amount of enzyme activity in 120 h (0.00, 0.02 and 0.00 units/mL in the broth medium which supplemented with tributyrin, Tween 80 and waste cooking oil, respectively), and during incubation time at 48 h showed 0.01, 0.11 and 0.00 units/mL of enzyme activity too.

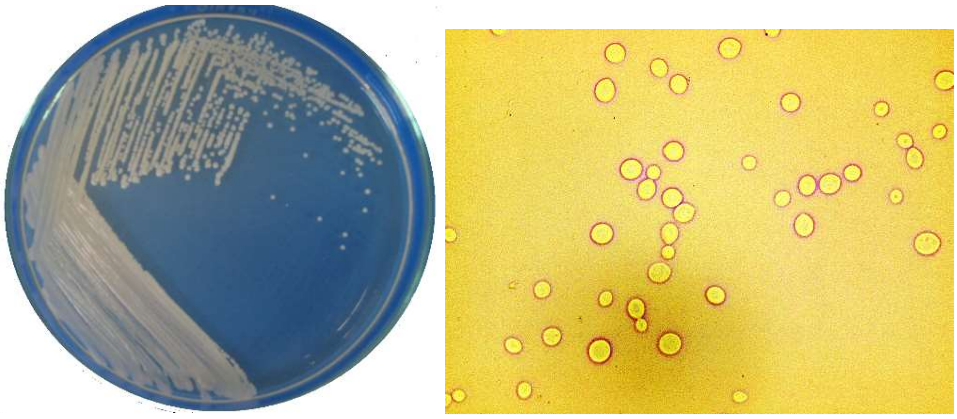
In 2014, Yalcin et al. proposed 14 strains were selected to lipase producing yeasts in the basal medium supplemented with 10 g/L tributyrin namely D3, D17, D24, D27, D30, D38, D40, D42, D44, D46, D56, D57, TAN10 and TAN46. There were showed 3.27, 2.86, 3.31, 3.05, 3.28, 4.25, 5.20, 3.50, 9.01, 7.89, 3.80, 8.87, 3.50 and 3.79 units/mL of lipase activity, respectively. For previously this reported, Wang et al. (2007) proposed that the lipase-producing yeast were belong to genus and species named as *Candida intermedia* YA01a, *Pichia guilliermondii* N12c, *Candida parapsilosis* 3eA2, *Lodderomyces elongisporus* YF12c, *Candida quercitrusa* JHSb, *Candida rugose* wl8, *Yarrowia lipolytica* N9a, *Rhodotorula mucilaginosa* L10-2 and *Aureobasidium pullulans* HN2.3 by cultivation in the broth medium contained 3% (w/v) Olive oil. Moreover, the optimal physical factors such as pH and temperature were different from each strains. However, the pH and temperature of all strains were generally between 6.0 to 8.5 and 35 °C to 40 °C. In addition, the various carbon sources such as olive oil, glucose and glycerol were studied to lipase producing from *Yarrowia lipolytica* KKP 379 and the optimal relative proportions of those substrates were evaluated in Mixture Design experiments. The medium contained 30 g/L glycerol and 1.92% (v/v) olive oil showed the best extracellular

lipase activity. It was reached 37.5 g/L while the original product is 1.0 unit/mL (Fabiszewska et al., 2015). The strain SPA4-2 was identified by molecular taxonomy and morphologically characteristic.

Identification of yeast

The morphological characteristic of yeast SPA4-2 strain presented pale pink, shiny, bulge and smooth edge colony (Figure 4A) and the cell description under blight field microscope at the magnification image 600X showed oval shape and multipolar budding, blastospore and true- psuedo mycelium cannot be observed (Figure 4B).

The molecular taxonomy of SPA4-2 strain at D1/D2 region of LSU rRNA gene showed 99.83% pairwise sequence similarity to type strain *C. liquefaciens* CBS 968^{T} (AF181515). Thus SPA4-2 strain was identified as *Cryptococcus liquefaciens*.



(A) (B)

Figure 4 Colony of yeast SPA4-2 on YM at 30°C for 48 h (A) and cell shape under bright field microscope at the magnification image 600X, after incubation at room temperature for 48 h (B).

CONCLUSIONS

A total of 73 isolates were screened for lipase production by plate assay method. Among these strains, 60 were observed a clear zone on tributyrin medium and 10 showed opaque zone on Tween 80 medium. The strain SPA4-2 was the best produced clear zone on tributyrin medium and showed sharply high value of enzyme activity index (EAI = 3.38) and the strain BP12 was widely produced opaque zone on Tween 80 medium and showed EAI value was 2.16, were cultivated in broth medium containing tributyrin, Tween 80, commercial palm oil and waste cooking oil. The strain SPA4-2 was the highest extracellular lipases activity was 1.53 units/mL in commercial palm oil medium and it was identified as *Cryptococcus liquefaciens*.

Suggestion

However, the strain SPA4-2 was efficacy to lipases producing under the optimize conditions. Therefore, the progress research about optimal conditions will be done later and SPA4-2 might be produced high amount of lipases and might be real used in the industries.

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