Can Tho University Journal of Science
website: sj.ctu.edu.vn

DOI: 10.22144/ctu.jen.2017.045

Isolation and identification of lipid-degrading yeast from wastewater of canteens and restaurants in Ninh Kieu district, Can Tho city

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Article info.

Received 26 Aug 2016
Revised 17 Nov 2016
Accepted 31 Oct 2017

Keywords

Candida, canteens and restaurants, lipid-contaminated wastewater, lipid-degradation yeast, lipid degradation, vegetable oil

ABSTRACT

High lipid (fats and oils) concentration contained in wastewater from restaurants and canteens released into environment directly, leads to polluted water and lock drainpipe systems. The lipid-degradation capability of lipid-degrading yeast was investigated for possible application in treatment of lipids-contaminated wastewater. Twenty-eight yeast isolates were isolated from 11 lipid-contaminated wastewater samples from many restaurants and canteens in Ninh Kieu district of Can Tho city, Vietnam. Fifteen isolates produced halo zones on Tween 20 agar medium and were determined to have ability of lipid-degradation, whereas three of them (B1, ST, Da2a isolates) were found to have the high ability of lipid-degradation by measuring development of halo zone diameter during 72 hours and identified by ITS-PCR (Internal transcribed spacer- Polymerase chain reaction) technique and DNA sequencing. After 7 days of cultivation, the rates of the degradation of lipid contaminated in wastewater by strain B1, ST, Da2a were 74.14%, 83.03% and 80.7%, respectively. The results of DNA sequencing were compared with GenBank database of NCBI by BLAST N software. The sequences from selected isolates showed high degrees of similarity to those of the GenBank references (between 97% and 99%). Isolates of B1 and ST were 99% of similarity with Candida palmioleophila and Meyerozyma guilliermondii, respectively. Da2a isolate was 97% of similarity with Candida tropicalis.

Cited as: Tan, N.N. and Diep, C.N., 2017. Isolation and identification of lipid-degrading yeast from wastewater of canteens and restaurants in Ninh Kieu district, Can Tho city. Can Tho University Journal of Science. 7: 27-32.

1 INTRODUCTION

Fats, oils and greases (FOGs) are released into the environment together with wastewater derived from the food processing industry, restaurants and kitchens. The main constituents of FOGs are animal fats and vegetable oils which are combination of glycerol and fatty acid. Lipids in wastewater are difficult to remove and degraded because they are hard to dissolve in water and they are known to inhibit methanogenic processes. For long time, accumulation of FOGs leads to pollution, locking of drainpipes and appearance of unpleasant odour. These problems always occur in big cities of developing countries.

Can Tho city is located at the central of the Mekong Delta, Vietnam with more than 1.2 million people living in 4 districts and 5 towns (General Statistics office of Vietnam, 2014). This city has many food processing industries, restaurants and canteens in universities and industrial zones to serve people, students and tourists. Therefore, a remarkable quantity of wastewater is released eve-

ryday with an amount of lipids into wastewater. Nowadays, microbial treatments in environment are interested because of environmental conservation, safety and high effectiveness. Many microorganisms isolated from soil and water samples are able to the ability to catabolize and remove lipids from wastewater. Hasanuzzaman *et al.,* (2004) reported that *Pseudomonas aeruginosa, Bacillus* sp. and yeast were researched lipid-degrading ability *in vitro*. *Bacillus subtilis* BN 1001 (Akiyama, 1991) had high lipase ability in degradation of lipid-contaminated industrial wastewater. Many other researches have performed with isolation, optimization and application of bacteria for treatment lipid-contaminated wastewater in ASIA such as Japan, China, India, Korea. In Vietnam, *Acinetobacter soli* was lipid-degrading bacterium isolated from wastewater by Diep *et al*., (2014).

Yeast strains are considered that have effective lipase ability (Hasanuzzaman *et al.,* 2004). They have abilities of adaption and tolerance in wastewater, quick increase in biomass and stable development which are potential treatment. Some studies were reported that *Yarrowia lipolytica* KF156787 (Bataiche *et al*., 2014), *Trichosporon asteroides* or *Candida rugosa* (Saxena *et al*., 2003) had a high lipase activity. The aims of this study were to (i) isolate the lipid-degrading yeast from wastewater samples from food processing plants and restaurants, (ii) study characteristics of colonies, shape and lipid-degradation index to select high lipid degradation strains, (iii) identify 3 yeast strains with the highest lipase ability.

2 MATERIALS AND METHODS

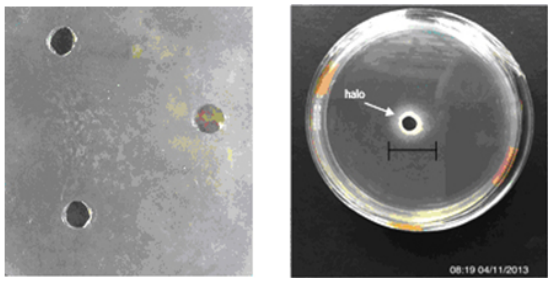
2.1 Sample collection

Eleven wastewater samples of 250 mL/sample were collected from wastewater drainage systems of many restaurants and canteens in Ninh Kieu district (Can Tho city, Vietnam). The sample of 1mL was diluted with 5 mL of distilled water, then 5% suspension would be enriched in 25 mL Yeast-extract Pentose-Dextrose (YPD) medium, incubated at 30^0C in 72 hours. The YPD medium included 10g Yeast extract, 10g pentose, 40g glucose/glycerol, 0.0001% chloramphenicol, pH=6 (Kurtzman *et al.,* 2010) and added 15g agar to YPD agar medium used. Isolation of yeast on YPD agar medium was conducted spread-plate method and incubated in 72 hours at room temperature with 0.1 mL diluted culture. Colonies were formed and sub-cultured on YPD agar plates by streak plate technique and re-incubated at 30^0C for 4 days to form single colonies. The pure isolates were tested by observation of cells with optical microscope (x400) and recorded characteristics of colony and cell.

2.2 Test of lipase ability

The primary of lipase test: The pure isolates were determined ability of lipase by streak plate technique on Tween 20 medium and incubated 72 hours (Diep *et al.,* 2014). The Tween 20 agar medium composed of 10g pentose, 5g NaCl, 0.1g $CaCl_2.2H_2O$, 20g agar, 1% Tween 20 at pH=6.5. The lipase yeast isolates from the primary test were inoculated in YPD broth composed of 10g Yeast extract, 20g pentose, 20g dextrose, 0.0001% chloramphenicol, pH=6.5 and incubated in 72 hours that was prepared for lipase assay later.

The lipase assay on Tween 20 agar medium: The plates of Tween 20 medium were made circular wells 6 mm diameter) as Figure 1, filled with 10 µl yeast suspension in YPD broth medium, incubated at room temperature. The diameter of each isolate was measured in the following periods: 24h, 48h and 72h (Diep *et al*., 2014). The experiment was completely randomized design with 3 replications. The data was recorded, calculated average and constructed the linear to find high lipase-activity isolates by using Microsoft Excel software 2010.



A B

Fig. 1: The well on Tween 20 medium agar (A) and the halo around well (B)

The lipase assay in real lipid-contaminated wastewater: The 10 mL cultures of high lipase-activity isolates were inoculated into 500 mL sterilized wastewater contained 1000-mL triangle flask. The treatments were incubated at room temperature and 140 rpm in a week. Lipid concentration in the treatments were measured by Adam Rose Gittle method at Advanced Laboratory of Can Tho University. The experiment was completely randomized design with 3 replications, data was recorded and LSD test at P=0.01 was used to differentiate between statistically different means by Excel 2010 software.

2.3 Identification of high lipase-ability yeast by PCR technique and DNA sequencing

The three highest lipid-degrading yeast strains from lipase assay were extracted DNA (Rogers and Bendich, 1989) and identified by PCR amplification of ITS/5.8S rDNA region with ITS1F (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4R (5'-TCC TCC GCT TAT TGA TAT GC-3') as forward and reverse primer (White *et al.,* 1990). The 50 µL reaction mixture consisted of 5U *Taq* Polymerase, 8µM of each desoxynecleotide triphosphate, 4 mM magnesium chloride, 1X PCR buffer 4, 1 µM of each primer and 50 ng DNA (White *et al.,* 1990). The thermocycling cycle was carried out with an initial denaturation at 95°C (10 min) followed by 30 cycles of denaturation at 95°C (60s), annealing at 55°C (60s), extension at 72°C (90s) and a final extension at 72°C (10 min) in C1000 Thermal Cycler (Bio-Rad) (Kumar and Shukla, 2005). Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Sequencing of the PCR products were performed in PHU SA Biochem (Vinh Long Province, Vietnam). The obtained sequences were aligned by using BLAST N analysis (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic tree was constructed by the Maximum Likelihood method using the MEGA software version 6.06.

3 RESULTS AND DISCUSSION

3.1 Yeast isolation, colony and cell characteristic

Twenty-eight yeast isolates were presented on YPD agar medium and isolated and recorded morphology of colonies and cells. Colonies of yeast were usually white or cream color, round, smooth/glistening or dry, covex or even umbonate as ST or B1 isolate (Fig. 2A). MT2 isolate was different from others, because the colony was red-cream color, smooth, covex with entire margin (Fig. 2B). Cells of MT2 isolate were globose, subglobose to ovoid shape, present or absent pseudohyphae (Fig. 3).

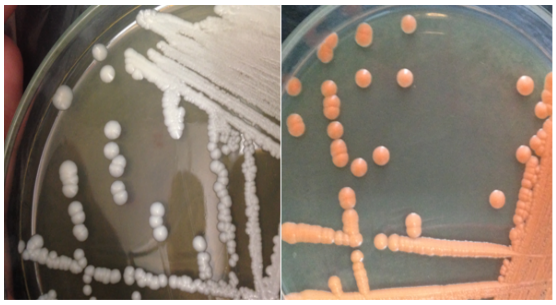


Fig. 2: The colonies of several lipid-degrading isolates ST (A) and MT2 (B) isolate(s) from wastewater on YPD agar plate

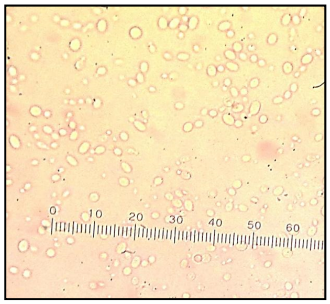


Fig. 3: Morphological cell of MT2 isolate (X400)

3.2 Screening for Lipid-Degrading Activities

After 3-day incubation on Tween 20 agar medium, 15/28 isolates having lipid-degrading activity were determined. They had presented halo zone occurred by degradation of lauric acid (fatty acid) created Ca^{2+} ($CaCl_2$) to precipitate of Calcium salt (Kurtzman *et al.,* 2010) (Figure 4).

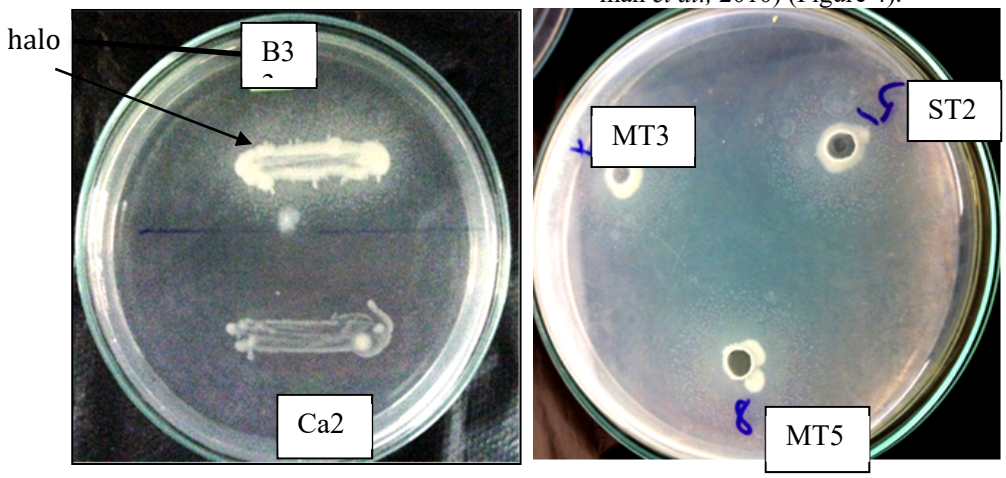


Fig. 1: The isolate having halo around their colonies

The isolates had good ability of lipid degradation in wastewater in comparison with the control (Table 1) with the development of halo (big halo diameter) in 72 hours. The 5 highest lipid-degrading isolates were described in Table 1. The three-of-which were chosen for further study. The three-isolates were compared the real disappearance of lipid in the wastewater (Table 2).

Table 1: Morphological characteristics of colonies and the analytical data of halo diameter during 72h (mm)

Isolates	Morphology	Size (µm)	17h	24h	41h	48h	65h	72h
B1	Coccus	1.5-3 x 1.5-4	10.67 a	13.33 a	19.33 a	21.33 a	26.67 a	28.33 a
ST3	Ellipse	1.5-3 x 1.5-4	10.67 a	13.67 a	19.67 a	21.00 a	27.00 a	28.00 a
MT5	Coccus	1,5-3 x 1,5-4	10.33 a	13.00 a	18.67 a	21.00 a	26.00 a	28.00 a
ST	Rhombus	2-3 x 3-4	8.00 ab	12.00 a	17.67 a	19.67 a	26.67 a	27.33 a
DA2A	Rhombus	2-3 x 4-5	8.00 ab	11.00 a	17.33 a	19.33 a	25.00 a	26.67 a
MT3	Coccus, rhombus	2-4 x 3-5	9.67 a	11.67 a	17.33 a	18.67 ab	24.33 a	26.67 a
PM1	Coccus	3-4 x 3-5	4.00 b	8.67 b	16.67 a	18.67 ab	24.33 a	26.00 a
ST2	Ellipse	4-6 x 5-8	5.67 b	9.33 ab	17.00 a	18.00 ab	24.67 a	26.00 a
ST2A	Ellipse	3-4 x 4-6	6.00 b	9.67 ab	16.33 ab	18.33 ab	23.33 ab	25.33 b
TD3	Coccus	3-4 x 3-5	4.67 b	6.67 b	13.67 bc	17.67 b	23.00 ab	25.33 b
MT2	Coccus, ellipse	3-6 x 4-8	6.00 b	8.33 b	14.67 b	17.33 b	22.33 b	25.00 b
KTX2	Ellipse	2-3 x 3-4	6.00 b	8.00 b	13.33 bc	15.33 bc	22.00 b	24.00 bc
HT2	coccus	2-3 x 2-3	5.33 b	8.00 b	12.67 c	14.67 c	22.00 b	24.00 bc
HL1	Coccus	2-3 x 2-3	4.67 b	7.33 b	13.67 bc	16.33 bc	21.33 b	23.67 c
CT3	Coccus	2-3 x 2-3	4.33 b	7.00 b	12.33 c	15.00 bc	21.00 b	23.33 c
		CV= 5.15%						

(Diameter of halo development = Total of halo diameter – diameter of well (6mm))

Means within a column followed by the same letter/s are not significantly different at p<0.01

The statistical analysis Table. 1 indicated that the development of halo zones and lipase activities of yeast strains correlated significantly (1% level) (F sample = 481.699> F.01 = 3.087), this showed that big diameter of halo demonstrated high lipase activities from yeast.

Table 2: Lipid concentration (mg/L) in wastewater after 7 days incubation with 3 isolates and control

Isolate	Lipid concentration (mg/L)
B1	9.51 a
ST	6.24 a
Da2a	7.33 a
Control (DC-C)	36.78 b
CV (%) =	5.15

Comparing with control sample, lipid concentration of B1, ST, and Da2a samples was decreased by 74.14% (27.27 mg/L), 83.03% (30.54 mg/L), and 80.7% (29.45 mg/L), respectively. The conclusion of lipid-degrading ability in real lipid-contaminated wastewater was ST > Da2a > B1 isolate.

3.3 Identification of yeast

The three isolates were chosen for identification and the DNA fragments of approximate of yeast 600 bp ITS region (ITS1F – ITS4R) were obtained from PCR and sequencing (Table 3). The characteristics of *Candida palmioleophila, Meyerozyma quilliermondii* and *Candida tropicalis* were similar records of Kurtzman *et al*. (2010).

Table 3: Phylogenetic affiliation of isolates on the basis of ITS genes sequences by using BLAST program in the GenBank database based on sequence similarity

Isolation	Relative species	Similarity (%)
B1	*Candida palmioleophila* Y-17323 (KJ705005.1)	99
B1	*Candida manassasensis* ATCC MYA – 4652 (HQ652050.1).	97
ST	*Meyerozyma quilliermondii* B-WHX-12-04 (KC544479.1)	99
ST	*Pichia quilliermondii* L2-8 (DQ663476.1)	99
Da2a	*Candida tropicalis* M267B (KP678451.1)	95
Da2a	*Candida tropicalis* M211A (KP675379.1)	96

The determination of nearest phylogenetic neighbor sequences for 18S - rDNA ITS gene sequence of the three yeast isolates by the BLAST search program showed that they grouped into two clus-

ters (Figure 5). Cluster A composed of two clusters: cluster of A1 and B1 isolates had 100% similarity with KJ705005 *Candida palmioleophila* strain Y-17323, and cluster of A2 and Da2a isolates had 98% similarity with KP675379 *Candida tropicalis* strain M211A while cluster B only with ST isolate had 98% similarity with KC544479 *Meyerozyma guilliemondii* isolate B-WHX-12-04. This result showed that three isolates were distributed in two clusters with two genera (*Candida* and *Meyerozyma*).

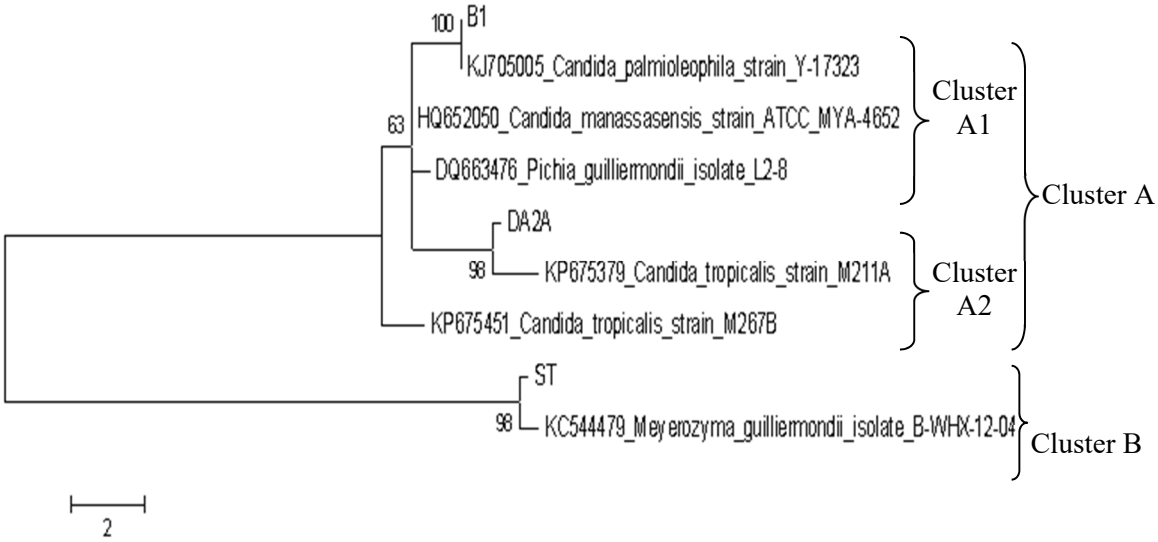


Fig. 5: Phylogenetic tree for ITS-gene sequences from 3 isolates by using primers (ITS1F – ITS4R) showing relationships between presented strains along with related sequences retrieved from GenBank

Microorganisms capable of degrading edible oil would be useful to solve the above-mentioned problems (Sugimori, 2009). Thus far, there are many reports on the microbial degradation of edible oil (Okuda *et al.,* 1991; Bednarski *et al.,* 1994; Wakelin and Forster, 1997; Suzuki *et al.,* 2001; Matsumiya *et al.,* 2007) from wastewater of restaurants and food processing industries. The coculture comprising yeast *Rhodotorula pacifica* strain ST3411 and *Cryptococcus laurentii* strain ST3412 was able to degrade efficiently even at low contents of nitrogen ({NH_4-N}=240 mg/L) and phosphorus sources ({PO_4-P}=90 mg/L). Besides that, they were the highest degradation rate observing at 20^{o}C and pH 8. (Sugimori, 2009).

4 CONCLUSIONS

From 11 wastewater samples in canteens and restaurants in Ninh Kieu district of Can Tho city, Vietnam, 28 isolates were isolated on YPD medium and 15 isolates on Tween 20 agar medium. Finally, 3 isolates having high lipid degradation ability were chosen to analyze the real degradation of lipid-contaminated wastewater and sequencing. The results showed that there were 74-83% of disappearance of lipid in the wastewater in a week by yeast. The three of determined lipid-degradation yeasts were *Candida palmioleophila, Meyerozyma quilliermondii* and *Candida tropicalis.*

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the helpfulness of students and technicians in the Environment Microbiology Laboratory, Biotechnology Research and Development Institute, Can Tho University, Vietnam.

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