

Identification of *Staphylococcus* strain CH1-8 and its oil-degradation

Sirilak Namwong¹, Somboon Tanasupawat²

¹Department of Biotechnology, Faculty of Sciences and Technology, Suan Sunandha Rajabhat University, Bangkok, Thailand.

²Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

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ABSTRACT

A lipase-and biosurfactant-producing, Gram-positive, aerobic and coccal bacterium designated as CH1-8 was isolated from Thai fermented fish. It grew optimally at 37 °C, pH 7.0, and in the presence of 0-5%, w/v NaCl. The results of phenotypic and chemotaxonomic properties including phylogenetic tree analysis confirmed that it was belonged to the genus *Staphylococcus*. The 16S rRNA gene sequence analysis revealed that strain CH1-8 was closely related to *Staphylococcus xylosum* ATCC 29971^T (99.11%), therefore it was identified as *Staphylococcus xylosum*. This strain gradually degraded various types of oils, sunflower oil, palm oil, soybean oil and rice bran oil, including cooking oil. During cultivation at room temperature for 35 days, lipase and biosurfactant activities of strain were 2 units/ml and 52 mN/m, respectively while the cleavage products were almost completely consumed. The maximal percentage of cooking oil hydrolysis by strain CH1-8 was 90% in the absence of NaCl and 60% in the presence of 10%, (w/v) NaCl. Therefore, the quality of wastewater from household will environmentally accepted after treated with strain CH1-8.

INTRODUCTION

The stable organic compounds in wastewater, waste oil either of petroleum or vegetable origin, have been discharged from food industries, restaurants and households and so on. High concentration of these compounds in aquatic environments are considered as serious types of hazardous pollutants because of decrease oxygen transfer rate into the aerobic process (Noh *et al.*, 2012; Becker *et al.*, 1999; Stoll and Gupta, 1997). Since the potential danger from oil pollution, mechanical fat separation or floatation have commonly used as procedures for oil and grease removing, however they are considered insufficient (Cruz *et al.*, 2010; Cammarota and Annajr, 1998; Stams and Oude, 1997). Therefore, the use of microorganisms and their metabolites for removing fat, oil and grease into miscible molecules have been studied i.e., the strains of *Pseudomonas fluorescens*, *Chromobacterium vinosum*, *Aspergillus niger*, *Humicola lanuginosa* (Yamane, 1987), *Rhizopus delemar* (Tuter *et al.*, 1998) and *Candida rugosa* (Chamorro *et al.*, 1998). These microorganisms can treat wastewater using various types of

organic matter as the substrate by the action of lipase and biosurfactants that reduced the interfacial tension between fluids having different polarities, such as water and oil (Shabatai and Daya-Mishre, 1992; Sigurgisledottir *et al.*, 1993). However, temperature and salinity that are the most influential parameters to effect the growth of the organisms in microbial enhanced oil recovery (MEOR) process at saline environment, have been discussed rather extensively (Kostka *et al.*, 2011; McInerney and Sublette, 2002). Therefore, lipase- and biosurfactant-producing halophilic bacteria occurred to thrive in saline environment, may solve such problems and they have not been reported as a wastewater treatment method. The objective of this study was to identify lipase-producing and biosurfactant-producing halotolerant bacteria from fermented fish (*pla-ra*) and to investigate the effects on biodegradation of cooking oil.

MATERIALS AND METHODS

Isolation and culture conditions

The bacterial strains were isolated from fermented fish (*pla-ra*) collected from Chumporn province, Thailand by spread-plate technique on Tween 80 agar plates [composed of (per liter): 100 g NaCl, 1 g Tween 80, 1 g peptone, 0.1 g CaCl₂.H₂O, pH 7.2] incubated at 37 °C for 1-2 weeks (Barrow and Feltham, 1993).

* Corresponding Author

Sirilak Namwong, Department of Biotechnology/ Faculty of Sciences and Technology/ Suan Sunandha Rajabhat University/Bangkok/Thailand
sirilak_pook@yahoo.com

Unless otherwise stated, the test strain was grown in liquid or on agar medium of the JCM medium No.377 agar plates [composed of (per liter): 100 g NaCl, 5 g casamino acids, 5 g yeast extract, 2 g KCl, 3 g trisodium citrate, 1 g glutamic acid, 20 g MgSO₄·7H₂O, 0.36 g FeCl₂·4H₂O, 0.0036 g MnCl₂·4H₂O, 20 g agar, pH 7.2] (Namwong *et al.*, 2005, 2009).

Characterization of strain CH1-8

The morphological and cultural characteristics were observed as described by Barrow and Feltham (1993). Biochemical testes were performed using the API Staph, API ZYM kits (bioMérieux). Strips were inoculated with API STAPH medium supplemented with 10% NaCl (v/v). DNAs were isolated and purified according to the method of Saito and Miura (1963). The 16S rRNA gene fragments were PCR-amplified with primers EB-20F (59-AGTTTGCCTGGCTC-39, positions 10–25 according to the *Escherichia coli* numbering system) and EB-1530R (59-AAGGAGGTGATCCAGCC-39, positions 1541–1525). The 16S rRNA gene fragments were separated by agarose gel electrophoresis and recovered by using the GenElute Minus EtBr spin column (Sigma). The sequences were determined using the BigDye Terminator Cycle Sequencing Ready Reaction kit (version 3.0; Applied Biosystems) in an ABI PRISM 310 genetic analyser (Applied Biosystems) with the following primers: EB-10F and EB-520R (59-ACCGCGG CTGCTGGC-39, positions 531–516), EB-530F (59-GTGCCAGCAGCCGCGG-39, positions 515–530), EB-1100R (59-AGGGTTGCGCTCGTTG-39, positions 1115–1100), EB-1110F (59-GCAACGAGCGCAACCC-39, positions 1099-1114) and EB-1530R. The sequences were multiply aligned with the CLUSTAL W program (version 1.81; Thompson *et al.*, 1997), then the alignment was manually verified and edited prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) in MEGA program version 2.1 (Kumar *et al.*, 2001). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Measurement of lipid degradation

Strain CH1-8 was selected for determination of cooking oil degradation. For inoculum preparation, it was cultivated in JCM no. 377 for a day at 200 rpm. For the analysis of its ability to degrade cooking oil, 5% (v/v) of the precultivated strain CH1-8 was inoculated in hydrolysis of lipid (HL) [composed of (per liter): 100 g NaCl, 10 g cooking oil, 5 g NH₄SO₄, 2 g KCl, 3 g trisodium citrate, 1 g glutamic acid, 20 g MgSO₄·7H₂O, 0.36 g FeCl₂·4H₂O, 0.0036 g MnCl₂·4H₂O, 20 g agar, pH 7.2] incubated at 37 °C for 35 days. During the cultivation, samples were taken for the analyses of cell growth, lipase activity, biosurfactant activity, lipid degradation and percentage of lipid hydrolysis. Lipid degradation was analyzed according to the method of Matsumiya (2007) using chloroform-methanol extraction method. The residual lipids was

performed using TLC aluminium sheet silica gel 60 (Merck, Darmstadt, Germany), with a mobile phase of hexane, diethyl ether and acetic acid (80 :20:1, v/v/v). The spots of lipids and degraded products were detected using saturated iodine steam.

Determination of percentage of lipid hydrolysis

Percentage of lipid hydrolysis was performed according to the method described by Freitas (2007). The remaining lipid was extracted using the mixture of chloroform - methanol (30:1). The dried sample was weighted and resuspended in 50:50 (v/v) of acetone in ethanol. The solution was tritrated with standard 0.02 M KOH with phenolphthalein as indicator. The percentage was calculated as follows:

$$\% \text{ hydrolysis} = \frac{(V_{\text{KOH}} \times M_{\text{KOH}} \times \text{MWm}) \times 100}{(W_t)}$$

where V is volume of potassium hydroxide solution required during titration; M is the KOH molarity (0.02M); MWm is the average of molecular weight of fatty acid (278.6 gmol⁻¹); W_t is the weight of the sample after air drying.

Lipolytic activity analysis

The lipase activity was examined by a spectrophotometric method using *p*-nitrophenyl palmitate as described previously (Kilcawley *et al.*, 2002). The assay mixture consisted of 900 µl of buffer [100mM sodium phosphate, NaCl (10%, v/v), Triton-X 100 (0.5%, v/v), adjusted to pH 7.0 with 1M NaOH], and 10 µl of 50mM *p*-nitrophenol palmitate in isopropanol. After incubation at 37 °C for 60 min, the reaction was stopped by adding 250 µl of 0.1M HCl. The activity of enzyme was quantified by UV spectrophotometry at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per min.

Biosurfactant activity analysis

Surface tension was measured and repeated at least three times using a du Nöuy ring-type tensiometer (Krüss, K10T) at room temperature. An average value was used to express the surface tension of the sample (Ghojavand *et al.*, 2008)

RESULTS AND DISCUSSION

Isolation of lipid-degrading bacteria

A total of 30 halophilic strains were initially isolated on Tween 80 agar medium supplemented with 10% NaCl (w/v) and tested for their ability to degrade lipids/fats. Amongst all the strains, four strains were found positive (CH1-8, PIS1-5B, PKD1-1B, PMH1B). Precipitation of free fatty acids with calcium (giving a white zone) was used as an indication to detect the bacterial activity for lipase production and lipid degradation. Strain CH1-8 shown the highest zone of calcium fatty acid was selected for further studies.

Characterization of strain CH1-8

Ahalotolerant strain CH1-8 was isolated from fermented fish (pla-ra) collected from Chumporn province, Thailand. This strain showed entire, translucent, convex and cream (0.95-2.55 mm in diameter) colonies on JCM no. 377 agar medium. During both exponential and stationary phase, cells were cocci and approximately 0.10-0.20 x 0.45-1.5 μm in diameter. The isolate grew in the medium containing a high concentration of NaCl (0-20%, w/v and optimally in 0-5%, w/v), at 15 to 45 °C (optimally at 37 °C) and at pH 6 to 8 (optimally at pH 7). Strain CH1-8 showed a turbid zone by emulsified oil on Tween 80 agar plate supplemented with 10% NaCl. The results of the standardized system for the identification of *Staphylococcus* species were summarized in Table 1.

Table 1: Characteristics of strain CH1-8 and *S. xylosus* ATCC 29971^T.

Characteristics	CH1-8	<i>S. xylosus</i>
Cell morphology	Cocci	Cocci
Gram staining	Positive	Positive
Catalase	+	+
Oxidase	-	-
Nitrate reduction	+	+
Alkaline phosphatase	+	+
Acetyl-methyl-carbinol	+	+
Arginine dihydrolase	+	+
Urease	+	+
Acid production from		
D-Glucose	+	+
N-Acetyl-glucosamine	+	+
α -Methyl-D-glucoside	-	-
D-Fructose	+	+
Lactose	+	+
Maltose	+	+
D-Mannose	+	+
D-Mannitol	+	+
D-Melibiose	-	-
Raffinose	-	-
Sucrose	+	+
D-Trehalose	+	+
Xylitol	-	-
Xylose	+	+

+, positive reaction; -, negative reaction.

Strain CH1-8 shared almost identical phenotypic properties with the type strain of *Staphylococcus xylosus* ATCC 29971^T (Spargser *et al.*, 2003; Trülzsch *et al.*, 2007; Tanasupawat *et al.*, 1992). A phylogenetic tree showing the position of strain CH1-8 in the genus *Staphylococcus* was reconstructed as shown in Fig. 1. The 16S rRNA gene sequence similarity values between CH1-8 and other recognized species of the genus *Staphylococcus*, were 97.51%-99.11% and it was closest to *Staphylococcus xylosus* ATCC 29971^T with 99.11% 16S rRNA gene sequence similarity. Based on the data from the API *Staph* system (acid production from carbohydrate, reduction of nitrate to nitrite, acetyl-methyl-carbinol production and enzymatic production (arginine dihydrolase, urease and alkaline phosphatase) and phylogenetic tree analysis, strain CH1-8 was highly affiliated to the type strain of *Staphylococcus xylosus* ATCC 29971^T; consequently, it was identified as *S. xylosus* (Essid *et al.*, 2007). Comparing to the

previous reports, thermohalotolerant, *S. xylosus* strain CH1-8 was able to produce lipases at room temperature and it may have the potential to play important roles in environmental applications i.e., degradation of oil waste (Mosbah *et al.*, 2005; Schmidt-Dannert *et al.*, 1996).

Degradation of vegetable oils and cooking oil by strain CH1-8

After 3 days of incubation on Tween 80 agar plate supplemented with 10% NaCl (w/v), strain CH1-8 formed turbid with emulsified Tween 80 around the bacterial colony revealing the presence of extracellular lipases. In Thailand, lipid-containing household waste usually comprises of various types of cooking vegetable lipids i.e., sunflower oil, palm oil, soybean oil and rice bran oil (PDC, Thailand).

Therefore, these popular vegetable oils were used as a sole carbon source for growth of lipase-producing bacteria. The growth of strain CH1-8 did not observed in the hydrolysis of lipid medium. Addition of vegetable oil (10%, v/v) and NaCl (10%, w/v) into the standard medium, the increase of turbidity and biodegradation were observed after 3 days cultivation. The percentage of degradation of palm oil, sunflower oil, soybean oil and rice bran oil, were 45, 33, 29 and 26 % respectively.

Based on the degradation ability of various types of lipids by strain CH1-8, it may cleave cooking oil to be fatty acid and glycerol. After cultivated in hydrolysis of lipid medium supplemented with 10% cooking oil, strain CH1-8 was capable to grow and hydrolysed cooking oil to be fatty acids and glycerol used as carbon source in order to enhancement of its growth. In agree with TLC analysis, the intensity of triglyceride and fatty acid spots gradually decreased within 35 days cultivation (Fig. 2b). In case of control, the degradation wasn't detected (Fig. 2a). During cultivation, lipase activity increased gradually during exponential growth phase (Fig. 3). In accordance with the previous reports, the extracellular lipases are generally inducible in the presence of different inducers i.e., olive oil, palm oil, oleic acid and Tween 80 (Shabatai, 1991; Sigurgisledottir *et al.*, 1993).

For the mechanism of lipid degradation, the emulsification was evaluated by the action of biosurfactants, surface-active substances, enhanced lipid degradation ability by decreasing the surface and interfacial tension of lipid and lipase (Noh *et al.*, 2012; Desai and Bannat, 1997). Therefore, biosurfactant production by strain CH1-8 was analyzed using surface tension measurement. After cultivation of this halotolerant strain under the optimum condition, the surface tension of cultured broth was reduced from 68 mN/m to 52 mN/m (Fig. 3) (water, 72 mN/m; Hydrolysis of lipid medium, 68 mN/m). In addition, the degradation of those lipids was high without temperature control indicated that strain CH1-8 has an efficiently degradation ability for various types of oils and cooking oil without the effect of seasonal temperature change. The results suggested that strain CH1-8 showed the potential to degrade the oil from household waste in presence of NaCl.

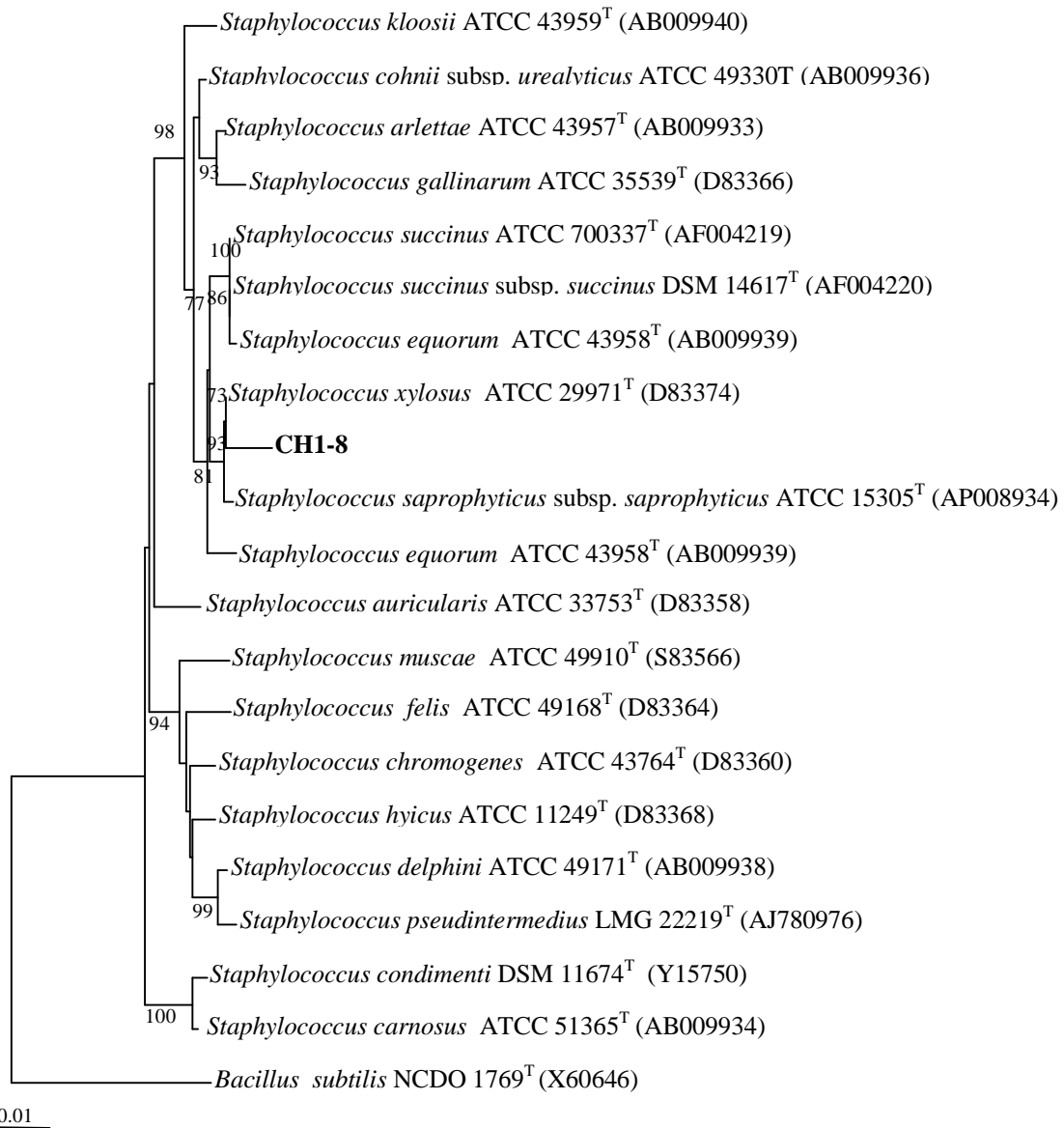


Fig. 1: Phylogenetic tree showing the relationships between strain CH1-8 and related *Staphylococcus* species based on 16S rRNA gene sequences. The branching pattern was generated by neighbour-joining method. Bootstrap percentages above 74%, based on 1000 replications, are shown at the nodes. Bar, 1 substitution per 100 nucleotide positions.

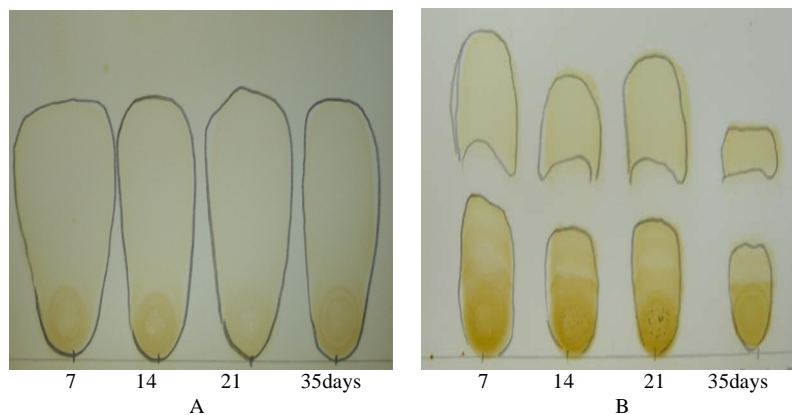


Fig. 2: TLC analysis of culture supernatant of strain CH1-8.

It was cultivated in HL medium at room temperature (b) and control (a). The solution for the mobile phase contained hexane, diethyl ether and acetic acid (80:20:1, v/v/v). The spots were detected using saturated iodine steam.

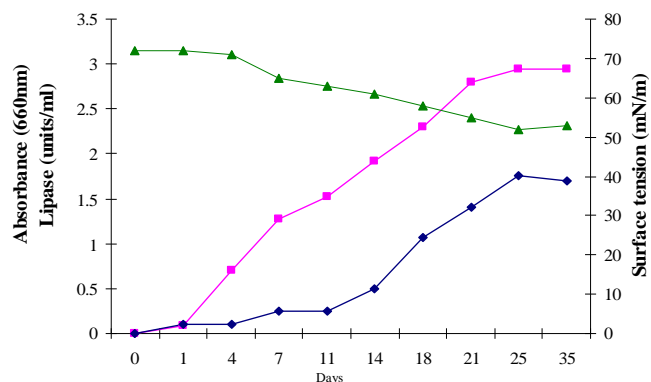


Fig. 3: Growth, lipase activity and biosurfactant production profiles of strain CH1-8. It was cultivated in HL medium at room temperature for 35 days. (▲), biosurfactant production; (■), growth; (◆), lipase activity.

Optimization of cooking oil degradation

In Thailand, lipid-containing household waste includes NaCl form reasoning i.e., fish sauce, soy sauce and salt (PCD, Thailand). Based on growth in the wide range of NaCl (0-20% NaCl (w/v) and optimal growing at 0-5% NaCl (w/v), strain CH1-8 might be potentiality to remove cooking oil in various of NaCl concentration. For enhanced oil degradation purposes, the effect of NaCl concentration and aeration were investigated. Strain CH1-8 was hence cultured in medium containing 0% and 10% (w/v) NaCl levels incubated with shaking (200 rpm) for 35 days at room temperature, and then TLC analysis of extracted lipids was observed as shown in Fig. 4b and 4d.

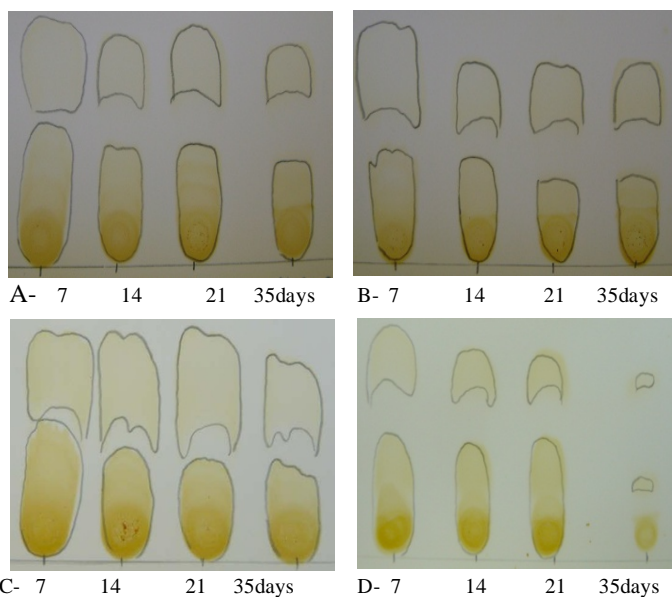


Fig. 4: TLC analysis of culture supernatants of strain CH1-8 cultivated in HL medium supplemented with (a,b) and without 10% NaCl (c, d) at room temperature and shaken at 200 rpm (b and d). The solution for the mobile phase contained hexane, diethyl ether and acetic acid (80:20:1, v/v/v). The spots were detected using saturated iodine steam.

The results indicated that the highest degrading activity was variable in the absence of NaCl with 91% degradation, however, the percentage of lipid hydrolysis was suppressed 29% of the maximally lipid cleaving in saline condition (Table 2).

Lipid degradation in the presence of NaCl (10%, w/v) was observed agreeing with effect of NaCl to its growth. In case of effect of aeration, media containing 0% to 10% (w/v) NaCl levels were used to culture this halotolerant with and without shaking at room temperature. The percentage of lipid hydrolysis by strain CH1-8 (Table 2) and its TLC analysis (Fig. 4a and 4c) indicated the higher lipid degradation was evaluated after incubated with shaking condition in the presence and absence of NaCl. Therefore, improvement of lipid cleavage ability may be by increase of interaction between microbial lipase, biosurfactant and lipid (Noh *et al.*, 2012). In comparison with other methodologies, a sequence of many necessarily techniques was approved such as chemical addition, biological filter, constructed wetland and land application or air flotation with/without alum followed by biodegradation i.e., activated sludge process followed by a high rate settler (Reed *et al.*, 1998). However, our results reveal that the usefulness of a single-step process without primary treatment under aerobic condition i.e., no addition of lipase and biosurfactant, manifested more advantage. In addition, an accomplishing biodegradation over wide range of NaCl concentration took place at room temperature, is considered a low energy uptaking compared to other treatments that need higher temperatures (35°C or 50°C) (Broughton *et al.*, 1998; Martine, 1991).

Table 2: Percentage of lipid hydrolysis of strain CH1-8 cultivated in HL medium.

Incubation time (days)	Percentage of lipid hydrolysis			
	With 10% (w/v) NaCl		Without NaCl	
	Static	shaking	Static	Shaking
7	20.63	38.57	21.28	31.93
14	28.04	45	25	49.13
21	39.1	56	27	78
35	44.04	65	28	91

CONCLUSION

A lipase- and biosurfactant-producing halophilic bacterium, *Staphylococcus xylosus* strain CH1-8 as the representative strain for the treatment of lipid-containing household, exhibited high efficiency for degradation of various oils and cooking oil as a single carbon source for its growth. Besides, the removing process at room temperature without additional physical or chemical treatment was achieved. From the data obtained, they suggest that the application of *S. xylosus* strain CH1-8 in microbial enhanced oil recovery (MEOR) process might be useful for treating the lipid-containing wastewater at saline environment. Moreover, further studies into the optimum cultivation conditions to improve the bioreactor and use of immobilized enzymes/cells from strain CH1-8 may lead to enhance the biodegradation during wastewater treatment.

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