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Full Paper

A high-activity lipolytic yeast isolated from Sichang Island, Thailand

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Abstract: Using the hydrolysis of *p*-nitrophenylpalmitate as a substrate to screen for extracellular lipase activity, seventeen isolates of lipolytic yeasts were obtained from 15 soil samples contaminated with petroleum oil from Sichang Island, Chonburi province, Thailand. Isolate SRY14-3 exhibited highest extracellular lipase activity (2.3 ± 0.1 units /mL) when cultured at 30°C, pH 5.2 for 4 days. Optimisation of the culture media by inclusion of 1.40% sucrose, 1.17% yeast extract and 1% cotton seed oil as the carbon, nitrogen and inducing lipid sources respectively increased the extracellular lipase activity by 32.3% to 3.05 units/mL. The optimal pH and temperature for the activity of the crude extracellular lipase were pH 6.0 and 40°C respectively. The enzyme was stable (> 80% residual activity) at pH 3-9 and temperature of 25-70°C. Sequence and phylogenetic analyses of the D1/D2 domain of the large subunit 26S ribosomal DNA placed isolate SRY14-3 as *Aureobasidium pullulans* and provisionally within the *A. pullulans* var. *melanogenum* clade.

Keywords: lipolytic yeast, ribosomal DNA, *Aureobasidium pullulans*, Srichang Island, oil-contaminated soil

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INTRODUCTION

Lipolytic enzymes produced by microorganisms have received considerable attention with regard to their potential and actual biotechnological applications. Recently, lipases are widely used in diverse biotechnological applications, for examples in production of detergents and surfactants, textile and dairy industries, oil processing, preparation of enantiomerically pure pharmaceuticals, and production of biofuels such as biodiesel [1, 2]. Based on the total sale volume, lipases are considered to be the third largest commercial group of enzymes following proteases and carbohydrases [3].

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyse the hydrolysis of water-insoluble triglycerides to fatty acids, monoglycerides, diglycerides and glycerol. In addition, under certain conditions they are also able to catalyse reactions such as esterification and trans-esterification [4]. Growth conditions known to influence the synthesis and secretion of lipases by microorganisms are the availability of suitable carbon and nitrogen sources, incubation temperature, pH, surfactants, oxygen tension and the presence of activators [5]. Increased lipase production is a crucial step for industrial applications as this directly affects their availability and economic viability [6].

Although ubiquitous in plants, animals and microorganisms, lipases are mainly and most widely harvested for commercial preparations from microorganisms including bacteria, fungi, yeasts and actinomycetes [7]. Based on their multi-fold catalytic properties, easy extraction procedures and superior biosynthetic capability compared to lipases from plants and animals, microbial lipases are considered an important source of biocatalysts for industry [8]. Yeast and fungi have been found to produce cell-bound and secretory lipases; the latter are secreted into the culture medium [9]. Yeasts in particular are attractive since they are generally considered to be non-pathogenic and environmentally friendly, and have prolonged exponential growth phase and high substrate concentration tolerance. However, high-activity lipolytic microbes such as yeasts are still required [10].

Sichang Island, or Koh Sichang, is a small island located along the eastern coast of the inner Gulf of Thailand, twelve kilometers offshore from the town of Siracha in Chonburi province. It is considered an important place for fishing, tourism and recreation. The area has also been assigned as a deep anchorage area for tankers and cargo ships. Human activities on the island have resulted in contamination of soils along the coast by various kinds of waste including grease and oily wastes from coastal communities. Although research on lipolytic yeasts has been performed in some other conditions in Thailand [11, 12, 13], relatively little work has been done on lipolytic yeasts in a marine environment. Hence the objective of this study is to screen and isolate the lipolytic yeasts from oil-contaminated saline soil. The yeast with highest hydrolytic activity selected is then further studied by optimisation of different parameters for maximal lipase activity and identified by using molecular methods based on the phylogenetic analysis of D1/ D2 domain of 26S rDNA.

MATERIALS AND METHODS

Isolation of Lipolytic Yeasts

Fifteen soil samples were collected from petroleum-oil-contaminated sites near an oil storage depot at Sichang Island. Soil samples (approximately 10 g) were collected using a sterile spatula and a clean, dry and sterile plastic bag. One g of each sample was added into 50 mL of yeast extract medium and the mixture shaken at 200 rpm at 30°C for 48 hr. The suspension was allowed

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to settle and the supernatant was harvested. The supernatant from all the samples was pooled and serially diluted to 10^{-1}-10^{-8}. One hundred μL of each dilution was spread onto yeast extract medium agar supplemented with 1% (w/v) palm oil emulsified in 10% (w/v) gum arabic solution and 0.001% (w/v) rhodamine B. The plates were then incubated at 30°C for 4 days and the extracellular lipase activity was screened under UV light at 350 nm [14]. Yeast colonies which showed orange fluorescent halo were considered positive and selected for further characterisation.

The selected colonies were replated on yeast extract medium agar at 30°C for 3 days. Individual colonies were transferred to the lipase-inducing production medium (bacto-triptone 10 g/L, yeast extract 3 g/L, meat extract 5 g/L, NaCl 5 g/L, KH_2PO_4 7 g/L, palm oil 10 g/L and rhodamine B 0.01 g/L) and cultured aerobically with shaking (200 rpm) at 30°C for 4 days. The culture was then centrifuged at 10,000 rpm (4°C) for 15 min. The supernatant was harvested and used as crude lipase preparation.

The lipase activity was determined spectrophotometrically using *p*-nitrophenyl palmitate (*p*-NPP) as substrate. Solution A contained 0.71 mM *p*-NPP dissolved in 2-propanol. Solution B contained 0.4% (v/v) Triton X-100 and 0.1% (w/v) gum arabic. A mixture consisting of 1 part of solution A and 9 parts of solution B was freshly prepared and 20 μL of the crude lipase preparation was added to 180 μL of the mixture. After incubation at 37°C for 30 min., the lipase activity was determined at 410 nm using a spectrophotometer. A lipase-inducing production medium (LPM) alone (without yeast culture) and a commercial source of lipase in LPM were used as negative and positive controls respectively. One unit (U) of lipase activity was defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol (from *p*-NPP) per min. under the assay conditions [15].

Effects of Carbon, Nitrogen and Lipase Induction Sources on Extracellular Lipase Activity

For the selected yeast isolate, the effect of lipase-inducing lipid, nitrogen and carbon sources in the culture medium on the extracellular lipase production levels in terms of net lipase activity were evaluated sequentially by a univariate approach. The yeast was initially cultured at 30°C in LPM with 1% (v/v) palm oil and 0.5% (w/v) yeast extract as the carbon and nitrogen sources respectively, and one of the following oils, i.e. camellia oil, canola oil, rice-bran oil, corn oil, sunflower oil, jatropha oil, sesame oil, cotton-seed oil, olive oil, safflower oil, soya-bean oil, coconut oil or palm oil, was added at 1% (v/v) as a lipase inducer. After 96 hr the lipase activity in the culture medium was determined as previously described and the lipid that induced the highest lipase activity was selected and used thereafter. Next, the yeast was cultured as above with addition of 1% (v/v) of the optimal lipid and one of the following nitrogen sources was used in each culture: yeast extract, tryptone, soybean meal, corn steep liquor, ammonium nitrate or urea, each at 0.5% (w/v). Thereafter, the yeast was cultured as above with the optimal lipid and optimal nitrogen source to determine the optimal carbon source from the following: sucrose, glucose, xylose or molasses at 1% (w/v), or no added sugar.

Optimal pH and Temperature for Crude Lipase Activity and Its pH- and Thermo-Tolerance

The crude lipase preparation (culture supernatant) was used to evaluate the optimal pH for extracellular lipase activity, which was determined as above by hydrolysis of *p*-NPP. The pH of the reaction mixture was varied between 3-10 by addition of 0.1M citric acid-sodium citrate buffer, 0.1 M sodium phosphate or 0.1M glycine/NaOH as appropriate. The optimum temperature for lipase activity was determined in a similar manner by varying the reaction temperature, viz. 25, 37, 40, 50

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or 60°C (pH 6). To evaluate the pH stability of the crude lipase, it was incubated for 24 hr at the selected pH before the determination of residual lipase activity using *p*-NPP assay. The thermo-stability of the crude lipase preparation was evaluated by incubating the enzyme at the 30-90°C for 30 min. prior to the residual lipase activity determination.

DNA Isolation, PCR Amplification, Sequencing of D1/D2 Domain of 26S rDNA and (Mega) BLASTn Analysis

Isolation of yeast DNA was performed by boiling the cells in lysis buffer as reported previously [16] with slight modifications. The D1/D2 domain of the 26S rDNA was amplified with the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTT CAAGACGG-3') (Pacific Science) [17]. The amplified DNA was purified using a QIAquick PCR Purification Kit from QIAGEN.

The nucleotide sequence of the amplified D1/D2 fragment was directly sequenced from the purified PCR product [17]. Cycle sequencing was executed with NL1 and NL4 primers as separate forward (leading strand) and reverse (lagging strand) reactions using ABI PrismTM BigDyeTM Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA). The obtained consensus sequence was then searched for highly similar or identical sequences in the National Centre for Biotechnology Information (NCBI) GenBank database [18].

Phylogenetic Analysis

The obtained consensus sequence of a 574 bp fragment of the D1/D2 region of the 26S rRNA gene of the yeast isolate SRY14-3 and the identical and most similar sequences found within the NCBI GenBank database using the BLASTn and MegaBLASTn algorithms were aligned using ClustalW. The pairwise sequence divergence of the aligned 574 bp of the sequences was used to form an evolutionary distance matrix, corrected by the two parameter transformations [19]. A phylogenetic tree was then produced using the neighbour-joining method. The reliability of the tree was tested by non-parametric bootstrap support with 1000 replicates.

Data Analysis

Numerical data from independent repeats are presented as mean ± standard deviation (SD). Significance of differences between means was tested by Analysis of Variance (ANOVA) and Duncan's multiple range tests (DMRT).

RESULTS AND DISCUSSION

Isolation of Lipolytic Yeasts

A total of 38 yeast isolates were obtained from 15 samples of oil-contaminated soil, 17 of which were found to be positive for the production of significant extracellular lipase activity. These 17 isolates were selected, subjected to liquid culture growth and evaluated for extracellular lipase activity after culturing at 37°C for 4 days. All 17 isolates revealed varying levels of lipase activity in the culture medium, as determined by the *p*-NPP hydrolysis assay (Figure 1). However, only isolates SRY1-1 and SRY14-3 showed lipase activity over 0.1 U/mL, with SRY14-3 having the highest activity at 2.30 ± 0.10 U/mL. It was then selected for further characterisation.

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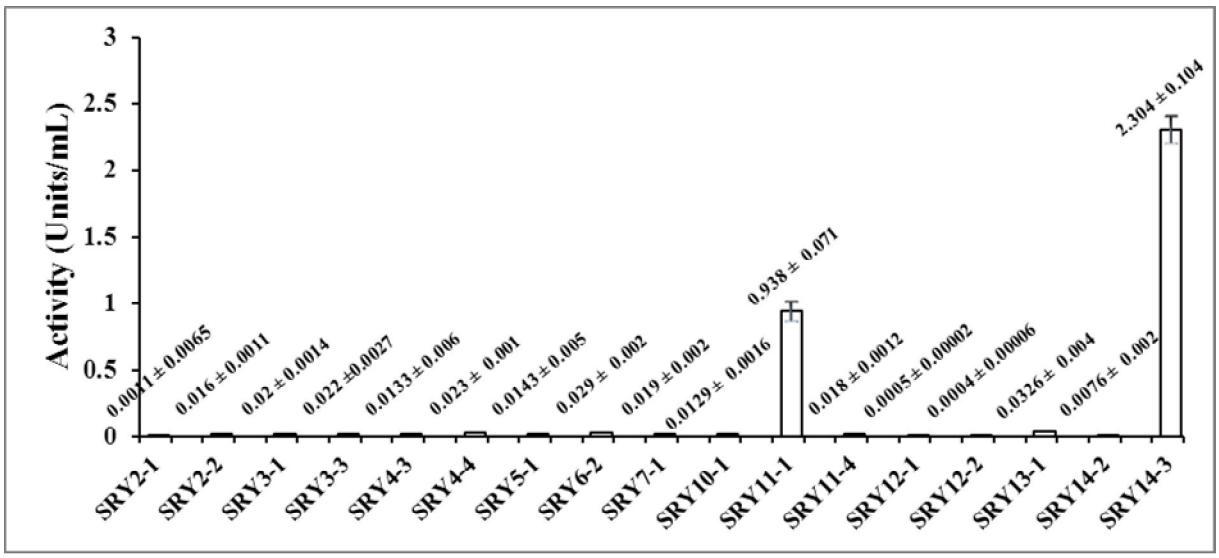

Figure 1. Extracellular lipase activity of different yeast isolates. Data are shown as mean ± SD derived from three independent assays.

Effect of Lipid Inducer on Extracellular Lipase Activity

Isolate SRY14-3 was evaluated for extracellular lipase activity after culturing in LPM pH 5 for 4 days with one of 13 different lipids as lipase inducer. The results are shown in Figure 2. In the absence of exogenous lipid (control) no significant lipase activity was detected. Although all the 13 tested oils induced lipase activity, only cotton-seed and palm oil induced an extracellular lipase activity of over 2 U/mL at 2.85 ± 0.12 and 2.43 ± 0.09 U/mL respectively (Figure 2).

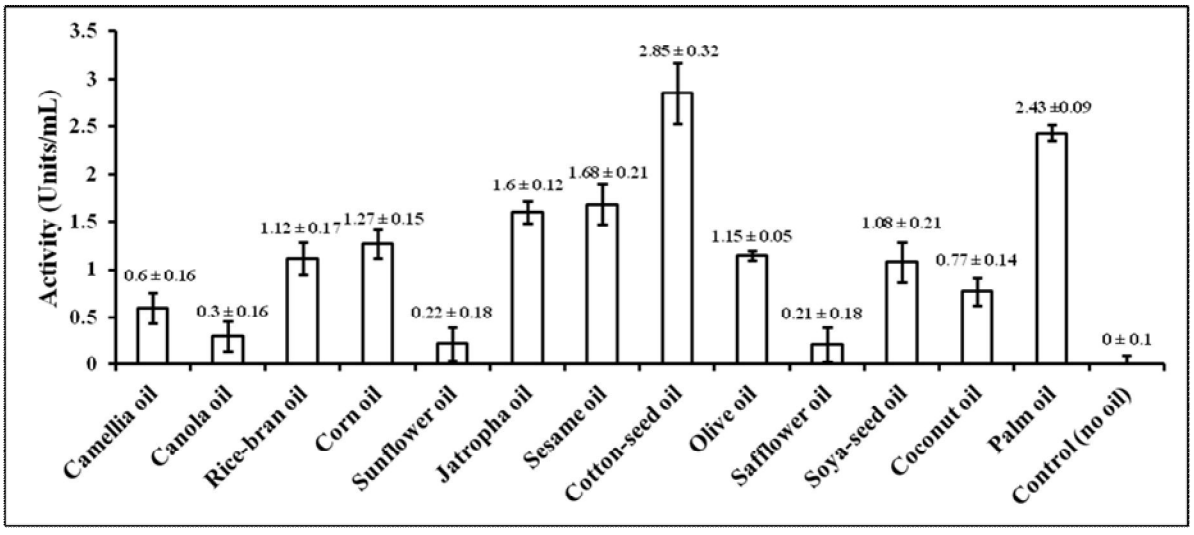

Figure 2. Effect of different lipid-inducing oil substrates on extracellular lipase activity of SRY 14-3. Data are shown as mean ± 1 SD derived from three independent assays.

In contrast, sunflower and olive oil were previously reported to be good inducers of extracellular lipase production by *Yarrowia lipolytica* [21], while vegetable oils such as sesame, groundnut, sunflower, palm, coconut and castor oils induced lipase in *Candida rugosa* [21]. However, the fact that all 13 tested lipids can induce extracellular lipase activity to some extent is

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consistent with the observation that lipases are generally produced in response to diverse lipid sources, such as oils, fatty acids, glycerol and Tween, in the presence of an organic nitrogen source [22].

Effect of Nitrogen and Carbon Sources on Extracellular Lipase Activity

Nitrogen source in the yeast culture media is known to affect the production of extracellular lipase activity [21]. In this study, with no exogenous nitrogen source, no significant extracellular lipase was detected (Figure 3a). Of the six different nitrogen sources evaluated, urea was ineffective and ammonium nitrate was very poor, whilst yeast extract was by far the best nitrogen source, attaining a lipase level of 2.75 ± 0.18 U/mL. This is consistent with the induction of lipase production in *C. rugosa*, where peptone and yeast extract were reported to be the best nitrogen sources for lipase production [23].

In addition to the nitrogen source, a suitable carbon source is required for optimal lipase production. From the five different carbon sources evaluated, sucrose gave the maximum lipase production of 3.05 ± 0.10 U/mL (Figure 3b), which agreed well with the reported lipase production by *Candida* sp. [24].

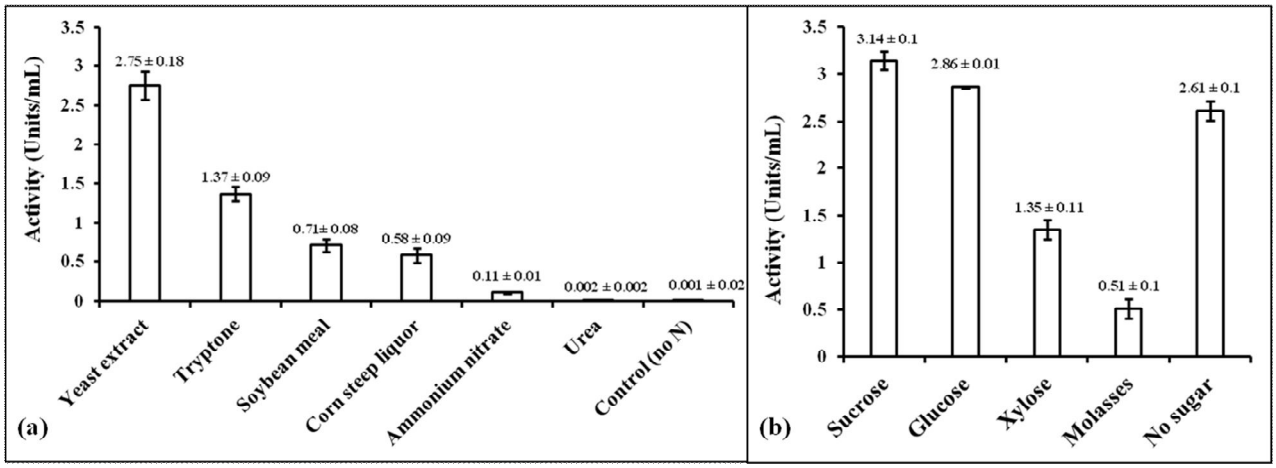

Figure 3. Effect of different nitrogen sources (a) and carbon sources (b) on extracellular lipase activity by SRY 14-3. Data are shown as mean ± 1 SD derived from three independent assays.

Optimum pH and Temperature for Lipase Activity and pH- and Thermo-Stability of Crude Extracellular Lipase

The maximum lipase activity was observed at pH 6, with a high (> 80%) lipase activity over a pH range of 3-7, but was alkali intolerant with < 60% activity at pH 8 and almost 0% at pH 9 or above (Figure 4a). In terms of the pH stability, the activity of the crude lipase was stable (residual activity > 80%) to a 24-h exposure at pH 3-7 and pH 9, but still retained >65% residual activity at pH 10. Thus, the crude enzyme was somewhat tolerant to alkali even though it was not active at alkaline pH. This pH optimum and stability are similar to those reported previously for most yeast lipases [5].

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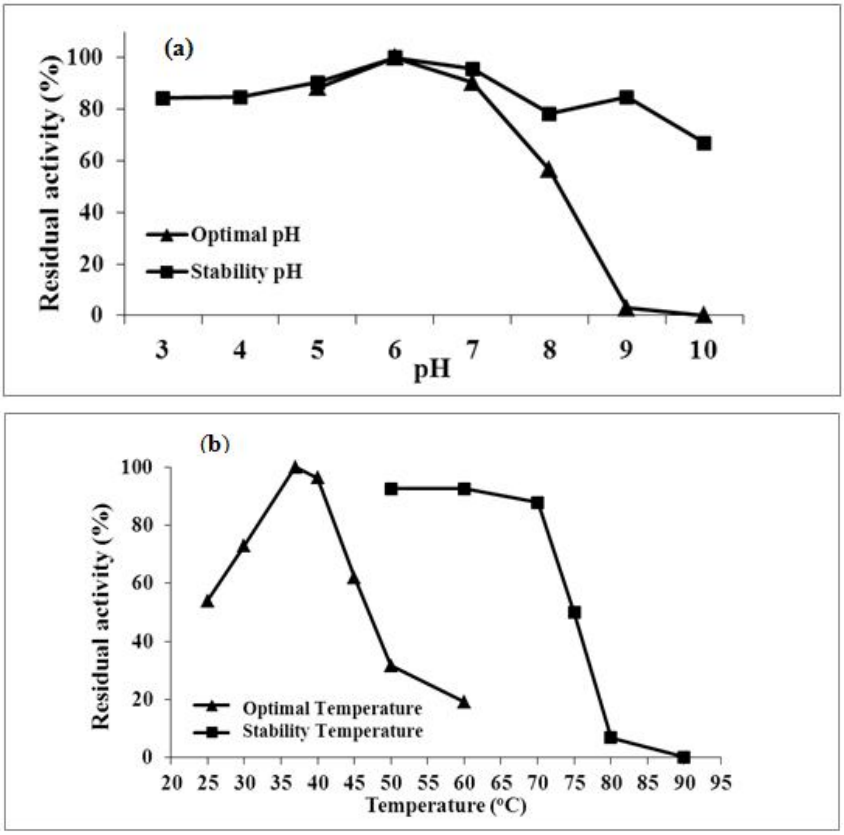

Figure 4. pH (**a**) and temperature (**b**) for the activity and stability of the crude yeast SRY 14-3 lipase. Data are shown as mean ± 1 SD derived from three independent assays.

As shown in Figure 4b, the optimum temperature for lipase activity was found to be 37-40°C, with much less activity at higher or lower temperatures. However, the thermo-stability in terms of residual lipase activity after exposure to the high temperatures for 30 min. at pH 5.3 was high with >85% residual activity at up to 70°C, but declined sharply at 80°C and 90°C with < 10% and 0% residual activity respectively.

Sequencing of D1/D2 Domain of 26S rDNA and Provisional Taxonomic Placement of Yeast Isolate SRY 14-3

The sequence of a 574 bp fragment of D1/D2 region of the 26S rRNA gene (GenBank accession code no. KF163060) was used to search for identical and most similar sequences within the NCBI GenBank database. From this search, no identical sequences were obtained, but 31 sequences with only two substitutions (572/574, 99.65% identical nucleotides) were found, and these were all designated as *Aureobasidium pullulans*, whilst of the 11 sequences designated as varieties, all were *A. pullulans* var. *melanogenum*. The most similar sequence not designated as *A. pullulans* was that of *A. leucospermi* strain CPC15099 (GenBank accession no. JN712554.1) [18] at 98.4% identity but over a slightly shorter sequence (559/568 nucleotides).

Selecting representative examples of the above sequences and those of different designated species, the sequences were aligned and checked and gaps removed as required. The pairwise sequence divergence between these representative sequences and that of SRY14-3 agreed with the sequence-identity-based placement of SRY14-3 within *A. pullulans* (not shown). The neighbour-

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joining-based phylogenetic analysis of these 574 bp aligned sequences, using *Elsimoe veneta* as the distant out-group, is presented in Figure 5.

In agreement with the sequence similarity discussed above, the phylogenetic pattern reveals with good bootstrap support that SRY14-3 nests within the *A. pullulans* group and also within the *A. pullulans* var. *melanogenum* clade, but separates from the clades containing other *A. pullulans* varieties and other related species (Figure 5). Thus, SRY14-3 appears to be a member of *A. pullulans* species and potentially belongs to *A. pullulans* var. *melanogenum*. From the guideline of Kurtzman and Robnett [17], where yeast strains with a nucleotide difference of less than 1% at the D1/D2 region of the 26S rDNA sequence are likely to be the same species, SRY14-3 is provisionally classified as *Aureobasidium pullulans*.

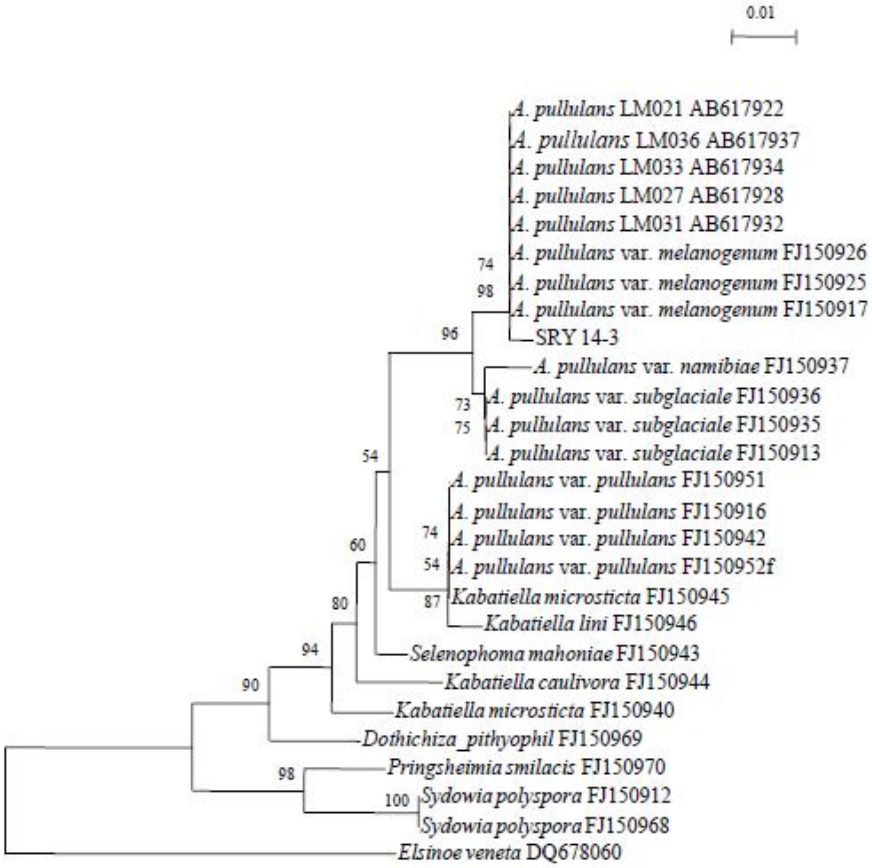

Figure 5. A neighbour-joining distance phylogenetic tree (Kimura's two parameter model), based upon 574 bp fragment of the D1/D2 domain of the 26S rDNA sequence of SRY 14-3 and related sequences. GenBank accession codes are given after the species name. Numbers indicate the bootstrap support as % from 1000 iterations. Scale bar shows the number of nucleotide changes.

CONCLUSIONS

A yeast isolate SRY 14-3 from Sichang Island was selected as a high extracellular lipase producer in the culture medium. It was classified from phylogenetic analysis based upon the sequence of D1/D2 region of the 26S rDNA, as *Aureobasidium pullulans* and likely *A. pullulans* var. *melanogenum*. Under optimised culture conditions, just over 3 U/mL of lipase in the culture medium was obtained. Its broad-pH activity, broad tolerance range and high thermo-stability are of

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biotechnological interest, although the substrate specificity should be confirmed for potential bioremediation applications.

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