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High-level expression and characterization of a lipase from *Trichosporon fermentans* Y3 and its application as an aquafeed additive for grouper (*Epinephelus coioides*)

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Abstract
A lipase gene was cloned from *Trichosporon fermentans* Y3, subcloned into pPICZαA, and then expressed in *Pichia pastoris* X33. The lipase (lipRT), which was obtained using a 50-L bioreactor, had maximum activity of 6000 U/mL. It had an optimum pH 8.0 and remained stable between pH 3.0 and 11.0, while its optimum temperature was 45 °C, maintaining more than 55% of optimum activity between 20 and 35 °C. To evaluate the potential application of lipRT as an aquafeed additive for juvenile grouper (*Epinephelus coioides*), fish with initial weight 13.31 ± 0.26 g were randomly divided into 12 sea cages and fed five formulated diets (labeled diets 1 to 4). Diets 1–4 were formulated to include 8% crude lipid and serial levels of lipRT (0 U/kg, 50 U/kg, 500 U/kg, and 1000 U/kg). The results showed that WG, FE, and PER of fish as well as ACH50, LSZ, and O_2^- production ratio in serum of fish on diets 3 and 4 were significantly ($P < 0.05$) higher than those on diets 1 and 2. The present data suggests that lipRT could improve the utilization of lipid and some nonspecific immunological parameters, and therefore could potentially be used as an aquafeed additive.

Keywords *Trichosporon fermentans* Y3 · *Pichia pastoris* X33 · High-level expression · lipRT · Juvenile grouper · Growth · Immunological parameters

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Introduction

Lipids are one of the important nutrients required for healthy fish growth (Borges et al. 2009), as they supply essential fatty acids, which are critical for fast fish growth (Sargent et al. 2002; Lopez et al. 2009). Dietary lipids also carry fat-soluble vitamins and serve as enzyme cofactors, as well as precursors of eicosanoids and hormones in fish (Watanabe 1982; Higgs and Dong 2000). Some previous studies have shown that lipids in diet could bring protein-sparing effect in some fish species (Torstensen et al. 2001; Chatzifotis et al. 2010; Karalazos et al. 2011; Liu et al. 2016). Further, dietary essential fatty acids are very important for fish disease resistance and immune response (Turchini et al. 2003; Subhadra et al. 2006; Jobling et al. 2008; Zhang et al. 2009; Jin et al. 2013; Liu et al. 2016). Therefore, low dietary lipid levels could decrease feed efficiency and weight gain (Luo et al. 2004; Lopez et al. 2009; Huang et al. 2016; Ni et al. 2016). Addition of exogenous lipase to diets has mainly been done for domestic animals and fowls with high growth performance (Polin et al. 1981; Al-Marzooqi and Leeson 1999, 2000). However, some recent studies have explored dietary lipase supplementation to improve growth, feed efficiency, and immune response of fish (Ran et al. 2015; Liu et al. 2016). Since fish are poikilothermal, while most farmed fish are raised in warm-water, with most carnivorous and omnivorous fish having acidic digestive environment in the stomach, exogenous dietary lipase should be able to withstand lower pH and maintain high enzyme activity between 20 and 30 °C. Unfortunately, most commercial lipases are not suitable because they are alkaline with higher temperatures required for optimal enzymatic activity.

Microbial lipases have been extensively used in the food and chemical industry due to their stability, efficiency, and substrate specificity (Domínguez et al. 2006; Singh and Mukhopadhyay 2012). Chen et al. (1993) isolated *Trichosporon fermentans* WU-C12, whose maximum lipase activity was only 128 U/mL at 30 °C. Although *Trichosporon* can produce extracellular lipase, the yield is not enough for large-scale use. Therefore, in order to achieve higher lipase yield, heterologous expression could be used to improve production and yield. *Pichia pastoris*, one of the most commonly used expression system, is suitable for the production of heterologous lipase. *P. pastoris* has many advantages over other expression systems such as high expression level and a powerful secretion ability (Chen et al. 1993). Apart from these, expression level of heterologous lipase genes in *P. pastoris* could be hundreds-fold higher than that in the natural host (Macauley et al. 2005).

In the current study, a lipase gene from *T. fermentans* Y3 was cloned and expressed in *Pichia pastoris* X33, followed by characterization of the biochemical properties of the lipase. The lipase was then evaluated in juvenile grouper (*Epinephelus coioides*) as an aquafeed additive for low lipid diets.

Materials and methods

Gene cloning, high-level expression, and detection of lipase activity

Genomic DNA and total RNA from *T. coremiiforme* Y3 (China National Research Institute of Food and Fermentation Industries Beijing, China) were extracted using Rapid Yeast Genomic DNA Isolation Kit (Sangon, China) and Fungal RNA Kit (Omega, USA), respectively. Based on the lipase gene (*tfl*) sequence (GenBank: AB000260) of *T. fermentans* WU-C12, two

primers TfF (5′-TCAGAATTCCAGGCCCCCACGGCCGTTCTC-3′) and TfR (5′-AGCT CTAGAACCGTAGAGATTAACGTC-3′) were designed. Next, *tfl* gene was amplified by PCR, and the PCR products digested with EcoRI and NotI, followed by ligation into the pPICZαA vector (Carlsbad, CA, USA) to generate pPICZαA-*tfl*. Finally, the recombinant vector pPICZαA-*tfl* was transformed into *E. coli* Top 10 (our laboratory's stock). Finally, positive pPICZαA-*tfl* clones were confirmed by DNA sequencing.

The pPICZαA-*tfl* recombinant plasmid was linearized and transformed into *P. pastoris* X-33 (Carlsbad, CA, USA) according to the manufacture's instruction. Recombinant clones were plated on YPDS plates containing 100 μg/mL Zeocin (Carlsbad, CA, USA). Transformed colonies were confirmed using PCR and sequencing. The clones from the YPDS plates were picked and cultured in flasks with shaking. The colony with the highest activity was isolated and used for high cell density fermentation in a 50-L bioreactor. Cell density and lipase activity were detected during the cultivation.

The lipase activity was detected by the following method: the substrate was prepared by emulsifying 15% (*v*/*v*) olive oil with distilled water which contains 2.5% gum Arabic as stabilizer. The pH and temperature for lipase activity determination were set at 9.0 and 45 °C, respectively. The enzyme activity unit (U) of lipase was defined as 1 μmol fatty acid liberated per minute (Wang et al. 2013).

Characterization of the lipase

The properties of the recombinant lipase were detected by the pH-stat method. The optimum temperature of the recombinant was analyzed from 20 to 60 °C. The thermal stability was also determined from 20 to 60 °C, with each temperature maintained between 15 and 120 min. The optimum pH of the recombinant lipase was measured at pH 3.0–10.0, with the pH stability determined by measuring the residual enzyme activities after incubation for 4 h at various pH (3–10) at 45 °C.

The effect of metal ions including Ca^{2+}, Cu^{2+}, Mg^{2+}, Na^{+}, K^{+}, Zn^{2+}, Mn^{2+}, and Fe^{2+} on the recombinant enzyme was determined, by incubating in 50 mM Tris-HCl buffer (pH 8.0) for 2 h. The residual activity was detected as described above.

Experimental diets and feeding experiments

To formulate the experimental feed, fish meal, soybean meal, chicken meal, gelatin, and shrimp meal were used as the main protein sources. Soybean oil and fish oil were used as the main lipid sources, while wheat meal served as the main carbohydrate source. The lipase was added serially at concentrations of 0 U/kg, 50 U/kg, 500 U/kg, and 1000 U/kg, together with 8% total lipid (Table 1). Solid ingredients were ground into suitable powder and filtered through a 120-μm meshed sifter. A pint-sized feed mixer was used to mix the five diet ingredients by the progressive enlargement method (Zhou et al. 2006) and a laboratory feed pelletizer was used to condense the ingredients, mixed with fresh water and oil, into 2-mm pellets. Next, the right amount of lipRT was diluted in fresh water and sprayed onto rotating feed pellets with a pint-sized mixer. Diet 1 was sprayed with same amount of fresh water (Robinson et al. 2002; Liu et al. 2012). All four groups of feed pellets were air-dried and sampled for biochemical composition analysis.

The feeding experiments were carried out in floating sea cages at Marine Biology Station of Shantou University (near 23°29′ N, 117°07′ E), Shantou, China. Juvenile grouper *Epinephelus*

Table 1 Formulation and proximate composition of experimental diets

Ingredient composition (g/kg)	Diets			
	1	2	3	4
Fish meal	350	350	350	350
Chicken meal	90	90	90	90
Gelatin	50	50	50	50
Soybean meal	205	205	205	205
Shrimp meal	80	80	80	80
Flour	110	110	110	110
α-starch	40	40	40	40
Fish oil	20	20	20	20
Soybean oil	10	10	10	10
Monocalcium phosphate	9	9	9	9
Bentonite	10	10	10	10
Compound vitamins	10	10	10	10
Compound minerals	10	10	10	10
Chloride choline	5	5	5	5
Vc phosphate	5	5	5	5
Lipase	0 U/kg	50 U/kg	500 U/kg	1000 U/kg
Proximate composition (%)	Content			
Moisture	10.52	10.35	10.64	10.53
Crude protein	48.89	48.94	49.03	49.07
Crude lipid	8.05	8.12	8.11	8.08
Ash	11.51	11.48	11.53	11.46

Vitamin premix (mg/kg): vitamin A, 4000 IU; vitamin C, 200; vitamin D3, 2000 IU; vitamin E, 200; vitamin K3, 6; vitamin B1, 7.5; vitamin B2, 16; vitamin B6, 12; vitamin B12, 100; folic acid, 2; pantothenic acid, 36; niacin, 88; Inositol, 100; biotin, 100

Mineral premix (mg/kg feed): magnesium sulfate, 5100; disodium hydrogen phosphate, 3200; dipotassium hydrogen phosphate, 8850; ferric citrate, 1100; calcium lactate, 12,100; aluminum hydroxide, 10; zinc sulfate, 130; copper sulfate, 5; manganese sulfate, 35; calcium iodate, 10; cobalt sulfate, 45

coioides were purchased from a marine fish hatchery at Yaoping County, Chaozhou, China, reared in three floating sea cages (3 m × 3 m × 2 m), and weaned from minced trash fish diets to formulated diet 1 as described in Table 1. During the 2-week acclimatization, fish were selected randomly into 12 experimental cages (1 m × 1.5 m × 1.5 m), each cage containing 25 fish, with initial weight of 13.31 ± 0.26. There were three replicates per each diet group. Fish were fed with the corresponding diets at 7 a.m. and 5 p.m. per day for a period of 60 days. Water temperature ranged from 24.6 to 28.2 °C, and salinity from 29 to 33‰, which were recorded daily before feeding. Dead fish were weighed so as to calibrate the feed conversion ratio.

Sampling, proximate composition, and statistical analysis

Prior to the start of the experiments, all fish were fasted for 24 h, after which 10 fish were randomly sampled for the initial analysis whole body composition, while another 30 fish were randomly sampled for initial body weight measurement. At the end of feeding course, the body weights of fish in each floating cage were measured, while 12 fish were randomly sampled, with 3 used for whole body composition analysis and 9 for body weight determination.

Blood was collected from the caudal vein of fish using sterile needle on a syringe with heparin. Leukocytes were isolated from the blood by the method of Lin and Shiau (2003), and respiratory burst activity was measured in terms of superoxide anion production (O_2^- production ratio) following the method described by Secombes (1997). Alternative complement

pathway (ACH50) activity was estimated using the method described by Montero et al. (1998) and the turbidimetric assay for lysozyme activity (LSZ) was carried out according to the method of Obach et al. (1993). The acid phosphatase (ACP) and alkaline phosphatase (AKP) of serum were determined according to the process described by Classics and Anderson (1962) and Bessey et al. (1946). The enzyme activity unit (U) of ACP and AKP was defined as 1 μmol phenol liberated from disodium phenyl phosphate per minute at 37 °C. Each parameter was tested in triplicate.

The biochemical composition of experimental diets and fish body was measured using routine methods (AOAC 1995), with moisture content of diets and fish measured by drying to a constant weight at 105 °C, protein content determined by the Kjeldahl method, lipid content by the Soxhlet method, and the ash content by combustion at 550 °C.

$$\text{Survival}\ (\%) = \text{final fish number}/\text{initial fish number} \times 100$$
$$\text{Weight gain}\ (\text{WG}, \%) = (W_t - W_0)/W_0 \times 100$$
$$\text{Feed efficiency}\ (\text{FE}) = (W_t - W_0)/F_i$$
$$\text{Protein efficiency ratio}\ (\text{PER}) = (W_t - W_0)/(F_i \times N)$$
$$\text{Lipid retention efficiency}\ (\text{LRE}\%) = (W_t \times P_t - W_0 \times P_0)/(F_i \times P)$$

where W_t and W_0 are the initial and final body weight; F_i is the feed intake; N is the protein contents of diet; P, P_0, and P_t are the lipid contents in diet, initial, and final fish body, respectively.

All data in this study were subjected to analysis of variance using Origin 8.5 and the level of significance was set at $P < 0.05$.

Results

Gene cloning and high-level expression

A 1700-bp putative fragment of the *tfl* gene was obtained by PCR. Sequence analysis of the fragment revealed that the ORF of *tfl* was 1671 bp, which encodes a protein with 556 amino acid residues. Analysis of the deduced amino acid sequence of TFL using NCBI protein BLAST showed that it shared 95% identity with *T. fermentans* WU-C12 lipases I (GenBank No. P79066.1) and 89% identity with *Galactomyces candidum* lipases I (Accession No. ALJ02547.1).

High cell density fermentation in a 50-L fermenter resulted in a continuous increase in the lipase activity and dry cell weight, reaching the highest value of 6000 U/mL and 186 g/L, respectively, after 144 h of cultivation (Fig. 1).

Characterization of the recombinant lipase

The activity and stability of recombinant lipase (lipRT) at different pH and temperatures are shown in Fig. 2. It was observed that lipRT maintained activity from pH 5.0 to 9.0, with the highest at pH 8.0. For pH stability, lipRT was stable for 4 h between pH 3.0 and 10.0 at 45 °C. For the lipase activity at different temperatures, it was observed that the optimal activity of lipRT was at 45 °C, which dropped sharply when the temperature went above 55 °C. Thermostability analysis showed that the activity of lipRT was very stable from 20 to 50 °C.

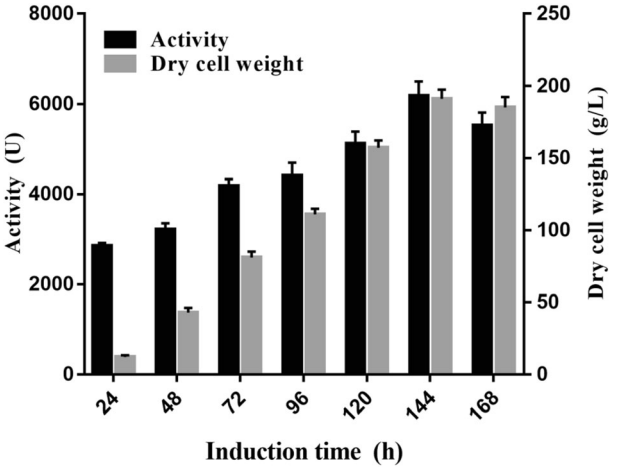

Fig. 1 Lipase production and cell growth during fed-batch fermentation in a 50-L bioreactor. The lipase activity was measured with a pH-stat (Metrohm) using olive oil as substrate at pH 8.0 and temperature 45 °C. Dry cell weight was obtained by centrifuging 10-mL samples in a pre-weighed centrifuge tube at 8000*g* for 10 min and washed twice with deionized water, before allowing the pellet to dry at 105 °C to constant weight

The effects of several metal ions on the lipase activity are shown in Table 2. It was observed that the maximum enhancement (about 15%) of the lipase activity was in the presence of Ca^{2+}, while Zn^{2+}, Fe^{2+}, and Cu^{2+} inhibited the activity by 35%, 25%, and 20%, respectively. On the other hand, Na^{+}, K^{+}, Mg^{2+}, and Mn^{2+} had slight effect on the enzyme activity in this study (80–103%).

Effects of lipRT on feed utilization, growth, and immunological parameters of juvenile grouper

After a 60-day feeding trial, the survival rate of fish in the different groups was not significantly affected by the lipase ($P > 0.05$). As shown in Table 3, the WG, FE, and PRE of group 3 (500 U/kg) and group 4 (1000 U/kg) were significantly ($P < 0.05$) higher than group 1 (0 U/kg) and group 2 (50 U/kg), but there were no significant differences ($P > 0.05$) among groups 3 and 4. Moreover, the WG, FE, and PER of group 2 were not significantly ($P > 0.05$) higher than those of group 1. The LRE of groups 2, 3, and 4 were significantly ($P < 0.05$) higher than group 1. The moisture and ash contents of the different groups were not significantly ($P > 0.05$) affected by the different diets (Table 3).

As shown in Table 4, the ACH50, LSZ, and O_2^- production ratios in serum of groups 3 and 4 were significantly ($P < 0.05$) higher than those of groups 1 and 2. The ACP in serum of group 4 was significantly ($P < 0.05$) higher than those of groups 1, 2, and 3, while the AKP of the different groups were not significantly different ($P > 0.05$).

Discussion

Features of the *P. pastoris* expression system

The methylotrophic yeast *P. pastoris*, which is currently reclassified as *Komagataella pastoris*, has been used extensively as one of the expression systems for heterologous protein

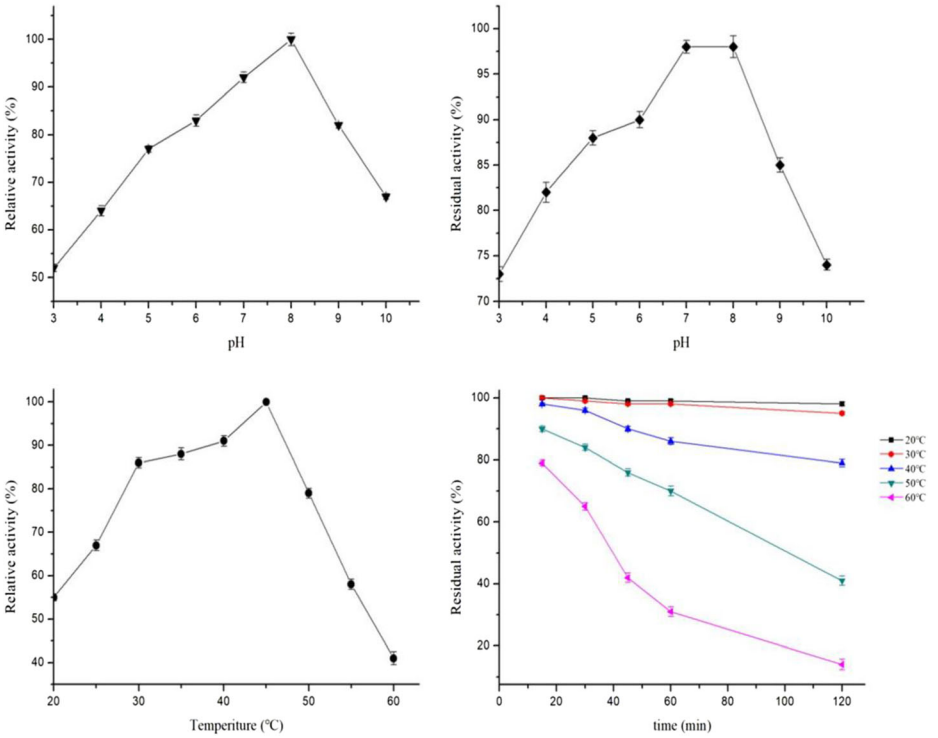

Fig. 2 Effects of pH and temperature on lipRT activity and stability. Optimal pH was determined by assessing the activity of lipRT at pH 3.0–10.0. The relative activity at different pH values was calculated by setting pH 8.0 as 100%. The pH stability was determined by measuring the residual enzyme activities after incubating lipRT at various pH (50 mM acetic acid-sodium acetic acid buffer (pH 3.0–5.0), 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 6.0–7.0), 50 mM Tris-HCl buffer (pH 8.0–9.0), and $NaHCO_3$ buffer (pH 10.0)) for 4 h at 30 °C. The residual activity was calculated using the sample without buffer treatment as 100%. The optimum temperature of lipRT was measured ranging from 20 to 60 °C. The relative activity at different temperatures was calculated by setting 45 °C as 100%. The thermal stability was studied by incubating lipase at various temperatures (20–60 °C) in pH 8.0 from 15 to 120 min. The residual enzyme activity was measured at 45 °C and calculated by using the samples without heat as 100%. All measurements were carried out in triplicate

Table 2 The effect of metal ions on lipRT activity

Metal ions (1 mM)	Relative activity (%)
Na^+	95.23 ± 0.71
K^+	103.24 ± 0.92
Mg^{2+}	80.58 ± 0.66
Zn^{2+}	35.34 ± 1.34
Mn^{2+}	90.56 ± 0.52
Fe^{2+}	75.21 ± 1.01
Ca^{2+}	115.34 ± 1.12
Cu^{2+}	80.72 ± 0.73

The effect of metal ions on lipase activity was analyzed by incubating enzyme samples for 2 h at room temperature in 50 mM Tris-HCl buffer (pH 8.0). The activity of recombinant was determined in the buffer with no addition of metal ions and set as 100%. Values are means ± SD of three determinations ($n = 3$)

Table 3 Effects of dietary lipRT composition on growth performance and feed utilization of juvenile *E. coioides*

Group	1	2	3	4
Survival (%)	96.00 ± 4.00	93.33 ± 2.31	94.67 ± 2.31	100.0 ± 0.0
WG (%)	152.7 ± 6.0a	157.3 ± 6.4a	178.9 ± 5.9b	187.6 ± 6.7b
FE	0.63 ± 0.02a	0.65 ± 0.04a	0.72 ± 0.06b	0.75 ± 0.05b
PER	1.29 ± 0.07a	1.33 ± 0.06ab	1.47 ± 0.08b	1.53 ± 0.05c
LRE (%)	38.45 ± 1.29a	42.61 ± 1.19b	49.85 ± 0.99c	50.01 ± 1.73c

Values are means ± SD of three groups of fish ($n = 3$). Within the same line, values with different lowercase letters are significantly different ($P < 0.05$)

production. *P. pastoris* has many advantages over other expression systems such as high expression level, powerful secretion ability, ease of genetic manipulation, and mature fermentation process (Daly and Hearn 2005; Macauley et al. 2005). In this study, we showed that the activity of a recombinant lipase (lipRT) increased from 20 to 6000 U/mL in *P. pastoris* X33 as compared to expression in *T. fermentans* Y3. It therefore suggests that heterologous expression of recombinant proteins in *P. pastoris* is a more effective method for improving the production of proteins of interest.

Properties of LipRT

The activity of the recombinant lipase (lipRT) was optimum at pH 8.0, which is similar with that from *Galactomyces geotrichum* Y05 (Yan et al. 2007) but different from some recombinant lipases from *Acinetobacter* (Kok et al. 1995; Snellman et al. 2002; Han et al. 2003). Remarkably, lipRT had stability over a wide range, from pH 3.0 to 11.0. To be used as a feed additive, it is very important that lipRT had stability at acidic pH, since it would have to pass through the acidic digestive environment in the stomach. The optimum temperature for lipRT activity was 45 °C, which is lower than that reported for lipases from *Galactomyces geotrichum* Y05 (Yan et al. 2007) but higher than that from *G. candidum* and *G. geotrichum* (25–40 °C) (Holmquist et al. 1997; Fernándeza et al. 2006). In addition, more than 60% of the lipase activity of lipRT remained at temperatures of 20 °C to 35 °C, which is the range for warm-water aquaculture. The activity of lipRT was inhibited by Cu^{2+} and Zn^{2+}, but was enhanced by Ca^{2+}, which is synonymous with lipases from *Acinetobacter* (Snellman et al. 2002; Park et al. 2009; Ahmed et al. 2010; Zheng et al. 2011) and *Pseudomonas aeruginosa* LX1 (Ji et al. 2010). Collectively, the data here suggests that the stability of lipRT

Table 4 Respiratory burst activity (O_2^- production ratio) of leukocyte and plasma lysozyme (LSZ), alternative complement (ACH50) activity, acid phosphatase (ACP), and alkaline phosphatase (AKP) in juvenile grouper *E. coioides*

Group	1	2	3	4
ACH50 (U/mL)	84.54 ± 7.35a	86.66 ± 5.27a	110.65 ± 10.35b	126.76 ± 11.06b
LSZ (U/mL)	3.86 ± 1.15a	4.45 ± 1.33a	8.54 ± 1.36b	9.67 ± 1.85b
O_2^- production rato	1.23 ± 0.21a	1.75 ± 0.11a	1.74 ± 0.14b	1.81 ± 0.13b
ACP (U/mL)	12.34 ± 1.25a	11.23 ± 1.24a	11.56 ± 1.65a	18.76 ± 3.15b
AKP (U/L)	146.45 ± 8.95	148.35 ± 7.25	147.46 ± 9.24	139.43 ± 7.67

Values are means ± SD of three groups of fish ($n = 3$), with 6 fish per group for respiratory burst activity, ACH50, lysozyme, ACP, and AKP. Within the same line, values with different lowercase letters are significantly different ($P < 0.05$)

at warm-water temperatures, as well as in the presence of acids and ions, makes it suitable for use as an aquafeed enzyme additive.

The effect of LipRT on growth and feed utilization of juvenile grouper

Groupers are warm-water marine fish (Pomeroy and Robert 2002) with an acidic digestive environment in the stomach. In this study, more than 60% of the lipase activity of LipRT remained between 20 and 35 °C, and with more than 70% residual activity at pH 3.0 for 4 h. Theoretically, dietary lipids should be efficiently hydrolyzed by LipRT in groupers' body. In fact, it was observed in this study that the WG and FE of fish given 500 or 1000 U/kg exogenous lipase (LipRT) in their feed were significantly ($P < 0.05$) higher than those of fish without lipase added to their diets.

Previous studies have reported that the optimum dietary lipid content for grouper (*E. malabaricus* and *E. coioides*) was 9% to 10% (Lin and Shiau 2003; Luo et al. 2005), while 8% dietary lipid in our study was at the lower level. The LRE data showed that supplementation with exogenous lipase efficiently improved lipid digestibility, providing more lipid products and energy to the body, which is comparable to adding more dietary lipids. The exogenous lipase also improved the protein sparing effect, as previously reported in some marine fish (Beamish and Medland 1986; Helland and Grisdale-Helland 1998; Torstensen et al. 2001; Kim and Lee 2005; Liu et al. 2016). In addition, the PER and LRE of the lipRT treatment groups were significantly ($P < 0.05$) higher than those without lipRT, suggesting that higher utilization of lipids results in a more efficient way of utilizing dietary proteins. However, weight gain is not only due to protein retention but also fat deposition (Gómez-Requeni et al. 2013).

The digestibility and utilization of protein from different source origin might be different by fish. Fish meal is recognized as one of the most effective protein sources for the growth of cultured fish. For diets of grouper *E. coioides*, *Cromileptes altivelis*, and *E. malabricus*, at least half of fish meal could be replaced by other animal protein sources such as animal by-product meals, poultry by-product meal, and so on (Millamena 2002; Shapawi et al. 2007; Wang et al. 2008). For diet of grouper *E. coioides*, 18.36% and 29.32% fish meal could be replaced by soybean meal and fermented soybean meal (Shiu et al. 2015). For juvenile grouper *Cromileptes altivelis* and *E. fuscoguttatus*, 20–30% fish meal could be replaced by soybean meal with phytase (Syah et al. 2006; Rossita et al. 2013). For juvenile grouper *E. fuscoguttatus* × *E. lanceolatus*, 50% fish meal could be replaced by soy protein concentrate without significantly affecting their growth and its body condition (Faudzi et al. 2017). In the formulated diets of this study, the ratio of other animal protein sources to fish meal was about 4:6 and soybean meal to total animal protein sources was about 2.5:7.5, which might not affect the growth performance of the grouper.

Dias et al. (1998) reported that when the lipid content increased to 180 g/kg in the diets of sea bass, significant weight gains were only observed at low protein level in diet, but not with a high protein levels. Similar results have been reported by Peres and Oliva-Teles (1999), where no protein sparing effect was observed in juvenile European sea bass with high protein level in the diets. The dietary protein level in the current study was about 49%, which is lower than the optimum protein levels of 50–55% for juvenile grouper (Shiau and Lan 1996; Usman et al. 2005; Tuan and Williams 2007), suggesting that the protein sparing effect should be more obvious in the present study. In fact, the higher PER and LRE observed in this study reflect better protein and lipid utilization in

the juvenile grouper. Thus, addition of LipRT could be used to obtain a better growth performance of grouper.

A previous study found that the LRE of grass carp *Ctenopharyngodon idella* fed with 2% dietary lipids was 148.86%, while those fed with 4% dietary lipids were 99.5%, which suggest that probably other nutrients might have been transformed into lipids in fish body when the dietary lipid levels were very low (Du et al. 2005). In fact, carbohydrates could be transformed into fat when lipids are lacking in fish (Gao et al. 2010; Xie et al. 2017). Takeuchi et al. (1978) reported that with lower lipid contents or higher protein contents in the diets of rainbow trout, a higher lipid retention rate was observed, suggesting that some part of the dietary protein was being converted to body fat. In the current study, the LRE increased with the addition of exogenous lipase (LipRT). With higher utilization of lipids, more lipids would be consumed to provide energy, with less proteins being transformed into body fat or energy provision. In this case, the protein sparing effect would be more effective, therefore resulting in significant improvement in WG, FE, and PER after the addition of lipRT. One of the interesting findings in the present study was that the LRE upon adding 50 U/kg lipRT was significantly higher than that without lipRT, although there were no significant differences in terms of WG, FE, and PRE. This observation could probably be due to the fact that the addition of 50 U/kg lipRT only improved lipid utilization to a small extent, with the increased lipid content mostly transformed into body fat, for which reason there was no more protein sparing effect that would lead improved fish growth.

Ni et al. (2016) had previously shown that low levels of lipids could decrease the immunity of young grass carp *C. idella* by reducing antibacterial compounds and the antioxidant ability, thereby triggering inflammatory response. Furthermore, Liu et al. (2016) revealed that exogenous lipase supplementation could enhance the intestinal TJ barrier function and antioxidant status in young grass carp *C. idella*.

In juvenile grouper *E. malabaricus*, Lin and Shiau (2003) reported that the ACH50, O_2^- production ratio, and LSZ of fish on 12% dietary lipid were higher than those on 0–8% dietary lipid, which resulted in significantly higher WG and FE. As observed in the present study, after 60 days of feeding juvenile grouper with 500 or 1000 U/kg LipRT, the main immunological parameters (ACH50, LSZ, and O_2^- production ratio) were significantly higher than those without addition of LipRT. Similar positive correlation between growth and immune-related parameters in grouper has also previously been reported (Lin and Shiau 2005a, b). It can therefore be inferred from the current study that more effective lipid utilization in juvenile grouper improved immunity with the addition of LipRT, which could be another main reason for the improved growth performance of the grouper.

In a previous study by Liu et al. (2016), it was revealed that the activities of digestive enzymes including chymotrypsin, trypsin, amylase, and lipase in the hepatopancreas and intestines of grass carp *C. idella* were significantly improved by adding exogenous lipase (1193 U/kg). Similar results were reported in another study (Ran et al. 2015) where the authors suggested that exogenous lipase promotes the growth of fish through improvement in digestive enzyme activities. Although intestinal digestive enzymes were not measured in this study, that the obtained result in this study suggested that the addition of the exogenous lipase could also promote growth of juvenile grouper by increasing digestion in intestines.

In summary, addition of exogenous lipase leads to higher lipid digestibility, thereby resulting in protein-saving effects, improved immune parameters, and digestive enzyme activities, which result in better growth of juvenile grouper; the basis for this study.

Given its optimal temperature and pH of LipRT, coupled with its stability in the presence of many metal ions, this recombinant lipase could potentially be used as an additive in warm-water aquafeed. Moreover, since addition of 500–1000 U/kg lipRT improved the growth performance and immunity of juvenile grouper, it suggests the suitability of lipRT for aquaculture. While the data from this study is interesting, more studies are required to further explore the use of this lipase in other fields of aquaculture.

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